CELLULAR MECHANISMS OF BETA RADIATION INHIBITION OF CORNEAL WOUND HEALING

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DEDICATION

To my parents,

Mr. and Mrs. Max M. Morrison,

for 28 years

of inspiration, encouragement and guidance.

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LIST OF ABBREVIATIONS

1. DNA ..... deoxyribonucleic acid
2. ER ..... endoplasmic reticulum
3. FAD ..... flavin adenine dinucleotide
4. GAP glyceraldehyde-3-phosphate
5. GAP-DH ..... glyceraldehyde-3-phosphate dehydrogenase
6. LDH ..... lactic dehydrogenase
7. LET ..... linear energy transfer
8. NAD .....nicotinamide adenine dinucleotide (DPN)
9. $NADH_2$.....reduced nicotinamide adenine dinucleotide
10. NADP..... nicotinamide adenine dinucleotide phosphate (TPN)
11. $NADPH_2$.....reduced nicotinamide adenine dinucleotide phosphate
12. PMN ..... polymorphonuclear leukocyte
13. RNA .....ribonucleic acid
14. SDH.....succinic dehydrogenase

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CELLULAR MECHANISMS OF BETA RADIATION INHIBITION OF CORNEAL WOUND HEALING

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Rabbit corneas were treated with 10,000 rads of beta radiation from a Strontium-90 ophthalmic applicator up to ten months prior to or immediately after surgical wounding. The clinical course of healing in the irradiated corneas was contrasted with the normal healing observed in the non-irradiated contralateral corneal wound. Tensile strength measurements were correlated with light and transmission electron microscopic studies of the corneal wounds. Histochemical enzyme studies were carried out to evaluate the radiation effects on enzyme activities induced by wounding the cornea. Isotopically labeled precursor studies using autoradiographic techniques were used to determine the number of cells synthesizing DNA and RNA in the wound area. Microphotometric measurements of Feulgen stained nuclear DNA were used to quantify the total DNA content per nucleus. Statistical interpretation of autoradiographic grain counts per cell were used to approximate the rate of RNA synthesis of the involved cells.

The inhibition of corneal wound healing by beta radiation was shown to involve many intracellular changes which had profound effects on the efficiency of the normal healing process. Beta radiation effects were found to be separate and different for the three major layers of the cornea.

A lack of tensile strength development in the irradiated corneal wounds appeared to be a result of a lack of synthetically competent cells, correlated with the absence of collagen synthesis in the wound area. Induced succinic dehydrogenase activity was reduced by the radiation effect, while 5-nucleotidase, $NADH_2$ diaphorase, and lactic dehydrogenase activities were unchanged.

Ultrastructural alterations resulting from the beta radiation treatments were correlated with inhibition of stromal collagen synthesis, and a decrease in RNA synthesis in all cells involved in the immediate wound. These findings together with the lack of regeneration of epithelial basement membrane and Descemet's membrane suggested that the protein synthesis capability of the cells had been inhibited by the beta radiation.

A reduction in fibroblastic proliferation was notably consistent with a reduction in cells synthesizing DNA and the total DNA content per cell nucleus.

Partial recovery of the healing capability was demonstrated when a ten month recovery period elapsed between the irradiation treatment and the subsequent surgical wounding.

The many complex effects of the radiation inhibition of the corneal healing phenomenon suggest that some comprehensive control mechanism

was inhibited by the radiation, rather than inhibition of a single critical metabolic or functional step in the normal repair process. The possibility of radiation interference with the mechanism that initiates wound repair and the potential damage to DNA synthesis and coding are discussed.

SECTION 1

GENERAL INTRODUCTION

Wound Healing

There is scarcely any question that wound healing and regeneration are important in the life and survival of a species. What constitutes the difference in connective tissues response between species which results in full regeneration of a limb for one species and cicatrization of an amputation in another species? This perennial question remains as a challenge since Eighteenth Century scientists first proposed to methodically characterize the schematic process of tissue regeneration in mammalian species.

A vast diversity of scientific knowledge has been accumulated to describe the complex phenomenon of wound healing. Various reactions between cells guide the process of healing. Many mechanisms are interwoven to control the course of movement and mitosis of involved cells, to eliminate the damaged cells through degeneration, and to determine from moment to moment the quantity and kind of intercellular material secreted and destroyed. The final result is manifested in the modulation of cell form, collagen and extracellular fabric, and eventually the process of wound contraction.

In skin wounds, epidermal regeneration begins immediately and progresses through three stages in the reconstruction of the corium (1). The first stage is the so-called "latent" period in which fibroblasts proliferate, migrate, and assemble. It is characterized by a low collagen content and low tensile strength of the wound during the first few days after injury. This latent phase, later renamed the "substrate" phase, has also been shown to be a period of intense biochemical activity (2). The second stage is characterized by fibroplasia which is indexed by a decline in the proliferation of fibroblasts, intense collagen synthesis, and the formation of fibers concomitant with increases in the tensile strength of the wound. The third stage involves maturation of these fibers, aggregation into bundles, and eventually contraction of the wound.

As yet the delineation and explanation of the interrelationship of these sequences of skin healing remain unfinished and challenging. Direct correlatives in the healing of the cornea provide an excellent opportunity to study the complexity of both normal and abnormal corneal repair, as well as the influence of various agents on the processes.

Corneal Wound Healing

The cornea is a unique tissue for the studies of wound repair as it is avascular and transparent. Anatomically it is arranged in several layers: (1) a stratified columnar epithelium, (2) an amorphous membrane (Bowman's), (3) a stromal layer, composed of collagen fibers imbedded

in a mucopolysaccharide ground substance that includes a sparce, but evenly distributed, complement of fibrocytes (keratocytes), (4) a second membrane (Descemet's), and (5) an endothelial cell layer.

The architecture and physiology that determines the transparency of the cornea also dramatically influences the repair processes that occur in this structure. These parameters include the functional integrity and capability of the endothelium, the strength, regularity, and arrangement of collagen fibrils of the stroma, the nature of the mucopolysaccharide ground substance, the integrity and physiology of the endothelial layer, and ultimately the state of deturgescence of these tissues.

Functionally, the three major layers of the cornea have separate and distinctly different roles in the process of healing. Briefly, the epithelial cells expand and slide to recover a defect in the epithelium. They must then multiply to re-establish the normal multiple cell-layered barrier to the environment. The stroma is important in providing strength to the cornea; therefore, during wound repair, stromal cell activity is predominantly that of synthesizing new protein and collagen. This requires morphological transformations of keratocytes and other invading cells into fibroblasts which synthesize new materials and proliferate. The endothelium is a highly differentiated cell layer whose prime importance is that of a selective barrier and pump that maintains the state of deturgescence of the cornea. The endothelial cells, therefore, are not required to multiply following injury,

but only to expand, slide over the defect, and re-establish the selective regulation of fluid flow through the cornea.

Considering the different functions of these major layers in the normal homeostasis of the cornea, it is appropriate to discuss the details of the healing process categorically by these layers.

Corneal Epithelium

The epithelium covers the corneal stroma acting primarily as a regulator of fluid and electrolyte exchanges and as a barrier to loss of metabolites.

Renewal

Cells of the basal layer of epithelium divide by mitosis about once weekly (3). These basal cells are the stem cells for the stratified squamous epithelial cells. Migration of the basal epithelial cells outward into the spinous layer occurs independently from the mitosis giving rise to the cell (4). The migration to the superficial layers follows mitosis. The epithelial cell turnover terminates ultimately in the superficial keratinized layers where the cells are finally desquamated into the tear film.

Repair of the epithelium

Defects in the epithelial layers are first covered by a process of spreading and migration by pseudopodial extension from the surrounding intact epithelial cells. Each cell can cover several times its normal area by expanding and flattening out. Recovering by lateral migration

begins approximately one hour after injury and concludes with complete covering of the stroma by at least one layer of epithelial cells. The initiation of lateral cell sliding apparently depends upon a breach of cell-to-cell contact, producing a loss of normal "contact inhibition" to cell migration (5).

Epithelial mitosis is depressed during the sliding and covering process due to some inhibitory effect of the mechanical insult. Mitotic activity returns after approximately six hours and accounts for the eventual replacement of the normal five to six cell layers several days, or even weeks, later (6).

The successful renewal of the epithelium and permanent recovering of the injury depends both on formation of an intact layer of epithelial cells and the re-establishment of a tight adhesion to the underlying stroma. Tight adhesion is dependent upon the integrity of the basement membrane and re-establishment often requires several days (7).

The ultrastructure of the epithelial cells changes during the lateral sliding and recovering process. Most prominent is the initial lack of a basement membrane in the basal cells during the first 36 hours after injury. At this time surface cells possess villus-like processes and cells in the depth of the wound interdigitate. The nucleoplasm of those cells with intact nuclei is irregular and irregularly shaped mitochondria appear as long, simple, organelles containing sparse internal membranes and almost no cristae. After 36 hours the mitochondria are increased in number, size, and complexity indicative of increased metabolic activity (8).

Importance of Epithelium in Stromal Repair

The successful recovering of the anterior corneal surface by epithelium has other implications in the normal physiology and repair processes that occur in the adjacent stroma. Optimum increases in tensile strength of corneal wounds has been shown to be dependent upon the presence of epithelium (9). Epithelium is necessary for the efficient incorporation of sulfate into mucopolysaccharides of the stroma (10).

Weimar has noted that the early stages of stromal healing are dependent upon the presence of epithelium. Apparently, a reconstituted epithelium is necessary for normal polymorphonuclear leukocyte (PMN) invasion from the periphery and keratocyte transformation into fibroblasts. This epithelial influence is thought to be mediated by a chemotactic substance released from the corneal epithelium upon injury (11).

Bowman's Membrane

Along the posterior of the basal epithelial cell layer of the cornea there is a basement membrane which separates the epithelium from the stroma. In certain species, in addition to the basement membrane, there is a region in which the collagen fibrils are randomly oriented (12). These two basic layers make up the Bowman's membrane.

In the posterior layer the random orientation is contrasted with the bundled lamellar architecture of the collagen fibril network found in the underlying stroma. Healing of Bowman's membrane is normally

considered part of the healing of the stroma since both layers are composed of comparable amounts of collagen. There is some question, however, as to whether the complete structure of Bowman's membrane ever regenerates. Bowman's membrane is found in the normal human cornea, but not in the cornea of the rabbit (13).

Corneal Stroma

The substantia propia, or stroma, of the cornea represents about 95 percent of the total corneal thickness. Its main function is to provide primary strength and body to the cornea structure. Fibrocytes (keratocytes) constitute about 95 percent of the cells of the stromal layer of the cornea. Cell division is very infrequent and keratocyte turnover is quite slow. Transplanted keratocytes labeled with radio-isotopes have been shown to persist in the stroma for a year or more (14).

Collagen turnover in the corneal stroma is extremely slow with a probable half-life of several years (15). Mucopolysaccharide turnover is much faster, however, with a biological half-life of approximately one month (16, 17).

Stromal repair

The first response to injury of the stroma is polymorphonuclear leukocyte and monocyte invasion within five to six hours (18). Morphological transformations of keratocytes adjacent to the wound edge begin within 24 hours. After some 60 hours, approximately 25 percent of the observed fibroblasts have been derived from the transformations of

keratocytes, 10 percent have been produced by cell division of these fibroblasts, and about 65 percent have been transformed from invading monocytes (19).

In healing of skin wounds, perivascular fibroblasts are thought to be responsible for the eventual replacement of tissue losses. Apparently, the major source of these fibroblasts is the proliferation and transformation of local connective tissue cells (20). Once mitotic division of these fibroblasts declines and fibroplasia begins, their role becomes one of synthesizing new collagen for replacement of the losses incurred in the wound. Intact fibroblasts appear necessary for the synthesis of new collagen although polymerization may actually take place extracellularly (21). Fibroblastic synthesis of collagen is indexed by: (1) uptake and subsequent release of sulfated compounds, (2) uptake and subsequent release of proline which later appears as hydroxyproline in the collagen fibers, and (3) the appearance of tropocollagen and the subsequent aggregation of tropocollagen into collagen fibrils in the extracellular substance adjacent to the cell surfaces (22).

Fibrocytes also have a rapid turnover of sulfated compounds; however, their role in the rebuilding of new collagen fibers and bundles is unknown (23).

Fibroplasia in the first two weeks of corneal healing is indexed by new collagen synthesis by the fibroblasts in the stromal area of the wound. New collagen fibrils are first found in a disorganized arrange-

ment, but by three weeks they are organized in a parallel fashion typical of the normal lamellar type architecture.

In severe stromal injury perilimbal vessels will invade the stroma when severe stromal edema is present. It is thought that the edema causes separation of the stromal fibers, reducing the normal compactness of the tissue and changing the state of deturgescence. Presumably, the reduction of tissue pressure and the action of some chemotactic factor then invites neovascularization (24). Stromal regeneration and restoration of the normal stromal thickness and state of deturgescence generally results in regression of these vessels, leaving only faint tracts observable upon biomicroscopic examination.

Endothelial lined lymphatic channels also develop in severely damaged stromas, connecting the more central stroma with the limbus (25). These lymphatic channels persist for some time after inflammation has subsided; however, they are only demonstrable with special histochemical techniques.

Scar formation in the cornea results in a concomitant loss of transparency in the areas adjacent to the scar. Electron microscope studies have shown that the normal collagen fibrils are replaced by fibrils with greater variation of diameters (12). Changes in both the normal architecture of the collagen fibers and in the amounts of extracellular mucopolysaccharides during the healing process are responsible for the loss of transparency in the wound area. Restoration of the normal lamellar arrangement, normal diameter of the collagen fibrils,

and normal mucopolysaccharide ground substance is required for renewal of corneal transparency.

Descemet's Membrane

An amorphous layer, called Descemet's membrane, secreted by the corneal endothelium lies between the posterior stroma and the endothelial layer. This membrane has stromal collagen fibrils embedded in the anterior portion adjacent to the stroma. Below the anterior portion is an organized portion, different from the lamellar structure of the stroma, and below the organized portion in the human cornea, is the posterior-most portion which has no regular organization (26).

Descemet's membrane contracts and rolls up at the free edges when it is torn. A new membrane is secreted slowly by the endothelium, requiring three months to attain one-half of the normal thickness (27).

Corneal Endothelium

Renewal

Mitotic figures are never seen in normal adult corneal endothelium. Regeneration of the endothelial cell layer apparently occurs by amitotic division at a slow turnover rate (28). Amitotic cell division requires approximately one hour and mitosis occurs only under unusual circumstances. The average life span of the corneal endothelium has been calculated to be 344 days in the rabbit.

Endothelial repair

Defects in the corneal endothelium are covered by endothelial cell migration beginning within 12 hours after injury (28, 29). Some mitotic divisions occur during the period required to cover the defect; however, the predominant activity is that of cell enlargement, spreading, and amitotic division persisting for several weeks.

Extensive damage to the endothelium may never be completely repaired resulting in an area of severely edematous stroma directly anterior to the area of incomplete endothelium and Descemet's membrane. A long-term swollen condition of the cornea invites complications such as neovascularization and non-specific inflammatory responses that may increase the severity of symptoms of the endothelial defect.

Biochemical Synthesis in the Healing Cornea

A variety of biochemical syntheses occur during the initial phase of corneal wound repair. Morphological transformations of keratocytes into fibroblasts can occur from one to two hours after injury, followed by dramatic increases in succinic dehydrogenase and 5'-nucleotidase activity in keratocytes and fibroblasts beginning approximately six hours after wounding (29). By two days an area approximately 200 mμ wide on either side of the wound edge contains fibroblasts and white blood cells intensely rich in both enzymes. Although cytochrome oxidase is not demonstrable in corneal wounds, another oxidase has been found to be active within the first 24 to 48 hours after injury and disappears between four and seven days (30).

Oxidative enzymes normally present in the cornea increase substantially after wounding (31). Nicotinamide adenine dinucleotide (NAD) and nicotinamide adenine dinucleotide phosphate (NADP) diaphorases both increase in the stromal and endothelial tissues following wounding of the cornea. Lactic dehydrogenase (LDH), malic dehydrogenase and alpha glycerophosphate dehydrogenase also increase following injury. Apparently, these increases are required as a result of the increased metabolism accompanying wound healing.

Protein and ribonucleic acid (RNA) synthesis begins within six hours after wounding, while deoxyribonucleic acid (DNA) synthesis is not apparent until approximately 12 hours after injury (32). Leucine incorporation into protein synthesis is especially high from 24 to 72 hours after wounding. Uridine uptake and incorporation into RNA synthesis peaks at about 12 hours and remains intense throughout 72 hours. Thymidine incorporation into DNA synthesis peaks around three to four days after wounding; however, protein, RNA, and DNA syntheses are all reduced by eight days of healing.

Mucopolysaccharide synthesis is elevated in the wound area beginning within 24 to 48 hours after wounding (33). Normal corneal stroma is complemented by at least three different types of polysaccharides: (1) chondroitin, (2) chondroitin-4-sulfate, and (3) kerato sulfate (34, 35). Kerato sulfate is thought to be specific for the cornea and has not been found in other tissues of the body. Although polysaccharide turnover is more rapid for the first one or two months of healing, the

total stromal content of sulfated polysaccharides remains subnormal throughout this period (36). Particularly noticeable is the gradual disappearance of kerato sulfate following wounding. The kerato sulfate reappears by approximately 30 days of healing, but is not present in normal quantities until after three months (37).

Collagen turnover in the cornea is extremely slow (38) and it is thought that new collagen is typically a soluble form which later thickens and becomes insoluble. New fibers are deposited over a period of months, but the exact rate of collagen synthesis is not well documented for the healing cornea. Corneal collagen is somewhat different from normal skin collagen in that the former contains more lysine and less threonine, serine, methionine, tryosine, and hydroxylysine than skin collagen (39).

Corneal Metabolism

Glycolysis

Enzyme activity determinations show that the Emden-Meyerhof pathway of glycolysis, the tricarboxylic-acid cycle, and the hexose monophosphate shunt (pentose shunt) are all present in the cornea (40).

In the corneal epithelium, 65 percent of the glucose is metabolized by the glycolytic pathway and 35 percent by the pentose shunt (41). In the stromal layer, however, oxidation of glucose appears to be entirely confined to the tricarboxylic-acid cylic pathway. Glucose exchange across the different cell layers of the cornea shows that the source

of glucose for corneal metabolism is predominantly through exchange across the endothelium. Estimates of glucose consumption by the cornea indicate a rate of 90 µg. per square centimeter per hour (40).

The hexose monophosphate shunt, which accounts for 35 percent of glucose metabolism in the corneal epithelium is thought to be linked to aerobic glycolysis via the reoxidation of the reduced form of NADP (e.g. NADPH) by lactic acid dehydrogenase (LDH) (41).

Other Metabolites

Lactic acid production in the complete absence of oxygen is about 10 µg. per hour per milligram of dry weight in the rabbit cornea (42). The lactic acid concentration in the stroma is approximately one-tenth that found in the endothelium and epithelium. It is thought that the epithelium probably generates and utilizes more lactate than the stroma, thus offsetting an activity level of LDH in the epithelium 200 times greater than the LDH activity in the stroma.

Glycogen is metabolized at a rate of 25 µg. per hour per milligram in excised cornea whenever the endogenous supply of glucose is depleted (43). Restricting the air supply to the cornea causes swelling and edema which appear to be related to glycogen depletion.

Respiration

The cornea respires only across the epithelial and endothelial surfaces with very little gaseous exchange with the limbal blood vessels. The oxygen flux from the atmosphere across the epithelium has been

calculated to be 7 µl. per square centimeter per hour (44). As one moves posteriorly the oxygen tension decreases steadily to a minimal level at the endothelium where the flux into the anterior chamber is quite small. This flux across the endothelium into the anterior chamber has been calculated to be only one-tenth that of the flux across the epithelium. The oxygen tension in the aqueous humor has been measured to be about 55 mm. Hg in the normal eye.

Oxygen utilization in the intact rabbit cornea has been measured at a normal rate of 8 µl. per hour per milligram dry weight (45). The rate of utilization is apparently independent of the oxygen tension in levels above that of normal air. Of this consumption the epithelium uses about 6 µl. per hour per milligram dry weight and the stroma and endothelium use the remainder.

The respiratory quotient of the cornea has been estimated to be unity; hence, the amount of carbon dioxide production in the cornea will be about the same as the oxygen consumption (45). Carbon dioxide is removed almost entirely by way of exchange across the epithelium and efflux into the air.

Beta Radiation in Ophthalmology

Beta Radiation Therapy

Beta radiation has been used in ophthalmology for over 50 years. Despite difficulties with depth dose dosimetry, construction and calibration of various applicators, and other technical problems, beta radiation is used for therapeutic treatment of many ocular diseases (46).

In a clinical study of 694 patients treated with beta radiation, 40 percent were treated with Strontium-90 applicators (46). Improvements in design and construction methods (47) have made Strontium-90 applicators the major source of beta radiation used in ocular therapy today.

Among the diseases of the eye treated with Strontium-90 beta radiation are: Mooren's ulcer, rosacea keratitis, pterygium, vernal catarrh, and vascularization of corneal grafts (48, 49). It has also been used for treatment of limbal tumors and malignant epibulbar melanomas (50). Therapeutic doses for these diseases range from 500 rads of beta radiation to 12,000 rads, with an average of 500 to 1,000 rads given four times at weekly intervals.

Ophthalmic Sources of Beta Radiation

Several sources of beta radiation have been used for ophthalmic applicators. These include: Radium, Radon, Radium D-E, and Strontium-90. Strontium-90 emits pure beta particles[1] with an energy of 0.54 Mev, and decays to a daughter nuclide Ytterium-90 that also emits a beta particle (energy of 2.2 Mev). Both Radium and Radon ophthalmic applicators have a gamma ray component in their radiation emission spectrum.

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[1] Radiological Health Handbook, U.S. Department of Health, Education, and Welfare: Office of Technical Services, September, 1960.

The advantage of using the pure beta emissions of Strontium-90 is based on the dosimetry and therapeutic index of the beta particles' shallow penetration of the ocular tissues. Depth dose determinations of beta radiation in ocular tissues have indicated that the dose at a depth of 1 mm. is only 50 percent of the surface dose, whereas the dose at 0.5 mm. is approximately 75 percent of the surface dose (47). Since the human cornea has an average thickness of approximately 0.5 to 0.6 mm., and the rabbit cornea has an average thickness of approximately 0.4 mm., a majority of the surface dose of Strontium-90 beta particles will penetrate beyond 2 mm. of ocular tissue.

Increasing the source strength of beta radiation applicators, giving higher surface dose rates, does not significantly raise the depth dose distribution of the beta radiation; instead, it effects the lateral dose distribution (51). Therefore, higher surface dose rates of a particular beta source result in isodose distribution curves that are larger in the area of tissue irradiated, but not significantly different in the dose rate at a specific depth in the tissue (52).

Dosimetry of beta particles in the presence of gamma rays becomes very complicated and less accurate than simple beta particle dosimetry. In addition, the greater penetration of the gamma ray component of many isotopic beta sources due to a lower Linear Energy Transfer (LET) makes undesirable exposure of the lens and other deeper ocular structures a therapeutic liability.

Sealed Strontium-90 applicators are, therefore, the most efficient isotopic sources for beta radiation therapy of the eye.

Beta Radiation Pathology of the Cornea

The effects of beta particle irradiation of the cornea have been classified as a function of the doses delivered to the tissue, subsequent changes occuring in the epithelium and stroma, followed by inflammatory reactions (53). The effects are cumulative and always become evident after a latent period which is inversely proportional to the dose received. Eventually, regenerative changes occur in all corneal structures; however, these never appear until after all the pathological changes are apparent.

The time sequence of the appearance of the various pathological effects of beta radiation on the cornea have been classified as follows: (1) early effects -- exemplified by edema and opacity, (2) delayed changes -- usually characterized by ulceration, perforation, and vascularization, and (3) late changes -- usually appear as re-occurrences of the original inflammatory lesions (54).

The threshold inflammatory dose of beta particle irradiation of the rabbit cornea (46) has been estimated at 22,500 rep,[1] however, the threshold dose in the human cornea has been documented to be 35,000 rep (53). Although ulceration may occur at 35,000 rep, consistent production of such lesions usually requires approximately 70,000 rep (54, 55).

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[1]The unit roentgen equivalent physical (rep) is an obsolete unit of absorbed dose, equal to 93 ergs per gram. This unit has been replaced by the "rad," which is defined as 100 ergs per gram of absorbed radiation. N.B.S. Handbook No. 66, Safe Design and Use of Industrial Beta-Ray Sources, U.S. Department of Commerce, 1958.

Re-occurrences of inflammatory lesions after several weeks or months is more frequent at the higher dose levels (usually above 50,000 rep). These late effects of beta radiation on the cornea often require several weeks or months to subside and many cases result in permanent scarring of the cornea.

Inhibition of Wound Healing

The complex phenomenon of wound healing involves so many diversified interactions of cells, morphological transformations, and new biochemical syntheses that the delicate balance of these interactions can be upset by a variety of agents and circumstances. The result is a delay or partial inhibition of the normal time sequencing of the healing process, and often complete inhibition of the repair process resulting in permanent and severe cicatrization and possible necrosis of the damaged tissue.

Scorbutic Wound Healing

Ascorbic acid (vitamin C) is known to be necessary for both the healing of wound tissue and the subsequent maintenance of the wound (56, 57). Three major changes are found in scorbutic wounds: (1) the endoplasmic reticulum is no longer found as elongated, flat, interconnecting channels, but rather becomes separate, vacuolated cisternae, (2) lipid accumulates in distinct deposits within the fibroblasts, and (3) non-banded filamentous material appears in place of the normal

collagen fibrils in the extracellular spaces (58). In addition, the rates of uptake and release of tritiated proline are slowed in the scorbutic wounds (59), indicating that the rates of synthesis of protein collagen is changed. It is thought that the scorbutic condition causes an alteration in the ultrastructural detail of the microsomes and endoplasmic reticulum that are responsible for hydroxylation of proline to hydroxyproline and subsequent synthesis of collagen protein.

Corticosteroid Inhibition of Wound Healing

Corticosteroid treatment of skin wounds produces a delay in the normal sequence of healing (60). The action of cortisone derivatives depresses fibroblastic proliferation, vascularization, and deposition of extracellular ground substances.

Similar observations in corneal wounds indicate that cortisone reduces fibroblastic activity (61), effects mitosis of epithelium (62), and reduces the tensile strength of corneal wounds when applied topically (63). Prednisolone reduced the development of tensile strength of corneal wounds when applied topically for two weeks following penetrating surgery (64); however, there was no effect on tensile strength if the prednisolone was withheld for the first ten post-operative days. Dexamethasone also retarded the development of tensile strength (65) and decreased the DNA synthesis in connective tissue cells (66) of the wounded cornea when applied during the first few days of healing. The effect of steroids on corneal wound repair appears to be proportional to

the dose, the frequency of application, and may well be cumulative since the effect was demonstrable when the steroid was applied two days prior to wounding.

Antimetabolites and Corneal Healing

Antimitotic drugs such as azathioprine (66) and 5-iodo-1-deoxyuridine (67, 68) have been shown to inhibit corneal wounds made by freezing. In these studies a correlation was noted between a low number of regenerating keratocytes and the extent of delay in healing. The mechanisms are similar in blocking thymidine incorporation into DNA synthesis. This is accomplished with 5-iodo-2-deoxyuridine by selectively blocking DNA polymerase through competitive inhibition of thymidine uptake and incorporation into DNA (69, 70).

Surgical Procedures Following Wounding

Central corneal wounds do not heal as fast as peripheral wounds because the more peripheral wounds are vascularized and invaded by cellular components of wound repair more easily than central wounds (71).

Closure of the eyelids following surgical injury to the cornea decreases the oxidative enzymes in the anterior cornea and delays cell division, but does not restrict the oxygenation of the repairing cornea severely enough to reduce the development of tensile strength of the wound (71, 31).

Covering of corneal wounds by a fornix-based conjunctival flap does not influence the tensile strength of the wound; however, removal of the epithelium drastically inhibits the corneal repair process (71).

Radiation Inhibition of Wound Healing

For over 35 years scientific reports have documented evidence of radiation inhibition of wound healing. Radium treatment of carcinoma of the cervix has caused problems with local healing of bladder fistulas (72) and irradiation of tissue prior to mastectomy has resulted in difficult healing and late degeneration of the irradiated tissues (73).

Although it has been suggested that very small doses of radiation may stimulate healing of animal skin wounds (74), it is well established that larger doses of 1,000 rads or more of x-rays will definitely inhibit skin wound healing (75). Even doses of 465 rads of x-radiation decrease the closure of skin wounds (76).

Radiation Effects on Corneal Healing

Various types of radiation have been shown to influence wound healing in corneal epithelium. Among these different radiations are Grenz rays, ultraviolet and x-radiation and beta particles.

Grenz rays are soft electromagnetic radiations with wavelengths of 1 to 4 A, which is shorter than ultraviolet radiation but longer than common gamma radiation. Grenz rays have been used in ophthalmology for superficial therapy because of their shallow penetration into eye tissues.

Grenz rays cause a temporary inhibition of mitosis at threshold dose levels (77). This inhibition of mitosis is followed by a rebound excess of epithelial cell mitosis.

Healing of corneal epithelium is similarly altered following exposures to ultraviolet and x-radiation (78) and threshold doses of beta radiation (79). Epithelial cell migration was not inhibited, unless doses reached levels high enough to cause nuclear fragmentation and indiscriminate destruction of epithelial cells.

Beta radiation effects on corneal healing

Doses as low as 2500 rep or approximately 23,000 rads of beta radiation have drastically inhibited penetrating corneal wounds (80). In these studies, 5,000 rep of beta radiation similarly inhibited corneal healing even when administered three months prior to wounding. Apparently, the beta radiation causes a delay in fibroblastic proliferation in the stromal portion of the wound as well as extensive stromal edema in the adjacent areas. The delay of fibroblastic activity and delay of stromal regeneration was evident six months later, concomitant with a residual edema surrounding the irradiated wound area. At higher doses of 27,000 rep fibroblastic proliferation was decreased and healing inhibited in corneas that remained normal in appearance for two years between the radiation treatments and surgical wounding.

The inhibitory effect of the beta radiation treatments apparently began to subside or decrease toward the end of these six month studies as healing eventually occurred. Thus, short term inhibition of epithelial

mitosis and prolonged inhibition of fibroblastic proliferation appear to be manifestations of a radiation-induced delay in the healing process and not a complete and permanent inhibition of corneal repair.

Recent studies of the effects of beta radiation on normal corneal endothelium (81), DNA synthesis during the first three days of corneal healing (82), and other observations of problems in corneal repair following combined therapeutic procedures involving local beta radiation have renewed interest in the radiation induced delay of corneal repair.

In addition, investigations of beta radiation suppression of the immune response to corneal xenografts have raised questions concerning effects on corneal healing of graft recipients (83). Complete inhibition of the normal immune rejection of intralamellar corneal xenografts was complicated by a residual edema and changes in mucopolysaccharide that were shown to be independent of any direct radiation pathology. Rather, the edema and mucopolysaccharide changes were thought to represent a problem in healing of the recipient's cornea (following the combined procedure of beta irradiation and surgery) and not a manifestation of immune rejection of the xenograft.

These studies suggest that more extensive work needs to be done on the site of beta radiation influence on the complex phenomenon of corneal wound repair. Specifically, more detailed studies are needed on the effects of beta radiation on cellular interactions, essential enzymatic activities, and macromolecular synthetic processes involved in corneal wound healing.

The following research was undertaken to delineate the sites of beta radiation influence on corneal healing and to gain insight into possible mechanisms of radiation inhibition of normal corneal wound repair.

SECTION 2

EXPERIMENTAL DESIGN AND GENERAL METHODS

The object of this research was to study the effects of beta radiation on corneal wound healing. Previous reports mentioned in the introduction have noted some inhibitiory effect of beta radiation on the process of healing of corneal wounds; however, no hypotheses have been established for the mechanism of this radiation induced inhibition.

Gross morphological and histological observations, along with tensile strength measurements of healing corneal wounds were used to estimate the magnitude and duration of the radiation inhibition. The initial experimental design also included biochemical observations and electron microscope examination of the fine structure of the cells involved in normal and irradiated healing corneal wounds. It has been documented that certain enzyme activities (29,30), nucleic acid, protein, and mucopolysaccharide syntheses (32) and other biochemical processes are increased dramatically in the wounded cornea. The cells of the different corneal layers, having separate functions, undergo different alterations in their ultrastructural organization during corneal wound repair. Such drastic changes in normal biochemistry and structure of these cells, observed only during wound healing, suggested that these physiological changes were essential to the success of the healing process in corneal wounds. It was reasoned

that the radiation would probably have some effect on one or more of these essential physiological processes if it inhibited and delayed the corneal healing phenomenon.

Beta radiation effects on the ultrastructure of cells involved in the healing cornea were correlated with the observed alterations in gross and microscopic morphology. Based on these findings, specific enzyme systems were evaluated by histochemical techniques to determine any radiation effects on critical metabolism in the healing cornea. These alterations further suggested that investigations into the different biochemical synthetic processes should emphasize evaluations of DNA and RNA synthesis. Radioautographic techniques were used to determine the effects of beta radiation on DNA and RNA syntheses in the repairing cornea.

The methods, execution, and results of these experiments are individually documented in the succeeding sections; however, the general materials and methods common to all these experiments are described below.

General Materials and Methods

Experimental Animals

One hundred sixty-five albino rabbits (*Oryctolagus aniculus*), weighing between 3 and 5 kg., were used in these experiments. The animals were purchased through the Animal Department of the J. Hillis Miller Health Center, Gainesville, Florida, from the Blueberry Rabbit Farm, New Port Richey, Florida. All the animals were maintained in

the animal quarters at the J. Hillis Miller Health Center during the experiments and all animals received identical care, feed, and treatment.

Facilities

All facilities, costs of materials, animals, and technical assistance for these experiments were provided by the Department of Ophthalmology, College of Medicine, University of Florida, Gainesville. Animal surgery facilities were provided by the Animal Department of the J. Hillis Miller Health Center, Gainesville, Florida. Histological facilities and the electron microscope were made available by the Research Service Unit of the Veterans Administration Hospital, Gainesville, Florida.

Surgical Wounding of the Cornea

Each animal received bilateral, penetrating surgical wounds in the central cornea. Preoperatively, 4 percent atropine sulfate ophthalmic drops were used to dilate the pupils. Intramuscular injections of pentobarbital sodium were administered 20 to 30 minutes prior to surgery. Four (4) to 5 cc. of 50 mg. per cc. aqueous solution of pentobarbital sodium was used as an anesthetic dose depending on the size and weight of each animal.

Using standard lid retractors for exposure, an 8 mm., non-penetrating incision was made in the center of the cornea to a depth of approximately one-half of the total thickness of the cornea. The

anterior edges of the incision were disected along the stromal lamellar plane, back away from the wound edge some 1 to 2 mm., providing a flap of corneal tissue on either side of the wound. Two 7-0 sutures were placed 5 mm. apart and evenly spaced from the center of the incision. Each suture was placed through both corneal flaps, perpendicular to and across the wound. The portion of the untied suture that bridged the wound was looped out of the way and the section was completed by insertion of a cataract knife at one end of the original incision, through the anterior chamber, and out at the far end of the original cut. A single upward movement of the cataract knife completed the section with a smooth cut that created a full penetrating wound at least 6 to 8 mm. in length. The sutures were then tied and the juxtaposition of the wound edges checked to insure uniform contact between the two edges of the closed wound.

Postoperative Care

Topical administration of 4 percent atropine sulfate and Neosporin^{R} ophthalmic ointment was maintained on a daily basis throughout the first three days following surgery. Thereafter, Neosporin^{R} was administered topically each day for the next four days of healing. Cases of infection or anterior synechiae were excluded from the studies.

Irradiation Treatments

All animals received bilateral wounds, however, only one cornea in each animal received beta radiation. Each animal served as its

own control since the observations made on each irradiated corneal wound ware compared with the normal wound healing in the non-irradiated contralateral cornea. The records of the identity of the irradiated and non-irradiated eyes in each animal and the dose administered were coded and all observations were made on a double blind basis.

Doses of 10,000 rads of beta radiation were selected, as this dose level was considerably higher than the doses previously reported to cause inhibition of corneal healing (80). Also, this dose is less than one-half the threshhold dose (35,000 rep) of beta radiation required to produce observable symptoms of radiation pathology of the cornea (53).

Of the 165 animals used in these experiments, 60 animals received 10,000 rads of beta radiation immediately after surgery, 60 animals received the radiation treatments two and one-half months before surgery, and 45 animals received the beta radiation ten months prior to surgical wounding.

Irradiation procedure

The eyelids were held open with standard retractors with the animals under pentobarbital sodium anesthesia and a drop of proparacaine hydrochloride ophthalmic anesthetic was administered topically. The beta radiation was administered by corneal contact with a Strontium-90 ophthalmic applicator having a surface dose rate of 104 rads of beta radiation per second (Atlantic Research Serial No. 232-Eye Therapy Applicator). Doses of 10,000 rads of beta

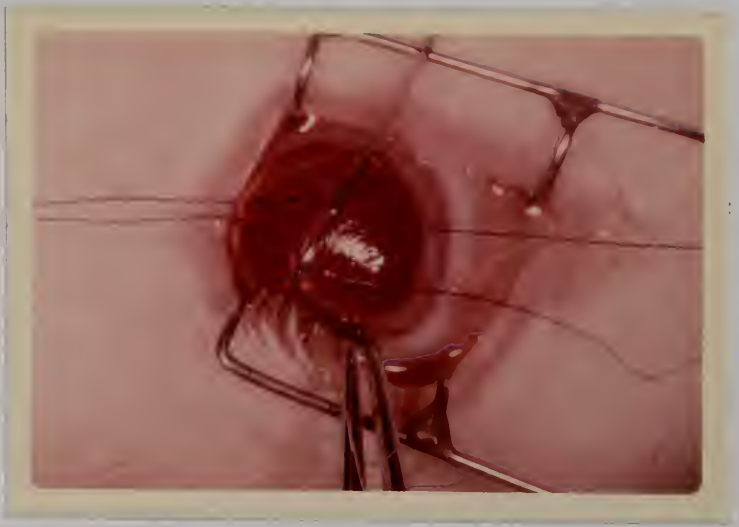
radiation required a 96 second exposure to the source. Other doses were calculated individually from the specified dose rate with a standard deviation of ± 6 percent.

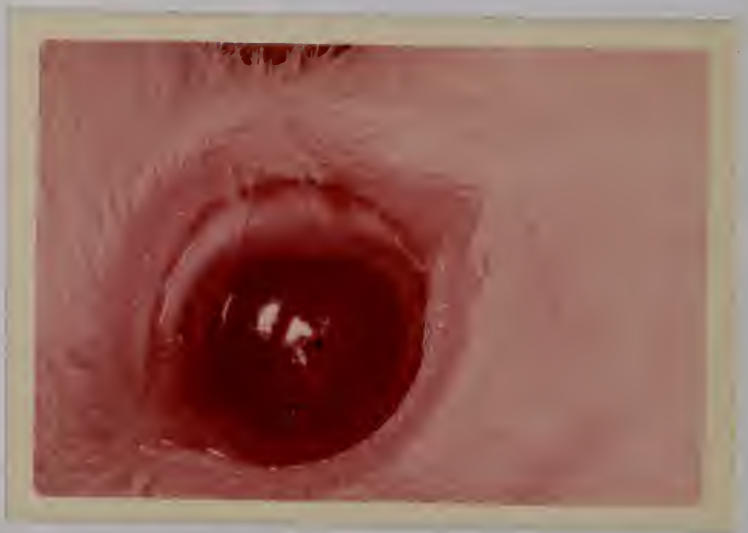
Clinical Observations

The clinical course of the corneal healing was followed in all of the experiments by both gross observations and detail studies using the biomicroscopic slit lamp. Photographs were taken with a 35 mm. camera apparatus designed for close-up pictures of the single eye. Pictures were taken at appropriate intervals to illustrate the progression of the healing process in the non-irradiated, control eye, as compared with the irradiated, contralateral eye in the same animal.

Figure 1. Technique of surgical wounding, graefe knife completing section. Note sutures placed superfically and looped out of the way.

Figure 2. Wounding complete, incision sutured.





SECTION 3

CLINICAL OBSERVATIONS AND TENSILE STRENGTH

The Clinical Course of Corneal Healing

Healing of the penetrating surgical wounds in the non-irradiated control eyes progressed normally. Within an hour after surgery, aqueous humor, leaking out, had formed in a fibrin clot in the wound, sealing off the anterior chamber. By six to twelve hours later, edema was noted in the local area around the wound. This produced an area of haziness along the wound perimeter; however, the remainder of the cornea remained clear.

Twelve to thirty-six hours was required in most cases for the epithelium to completely recover the wound. At this time the wound appeared as a narrow band of haziness with a hairline depression in the center, overgrown with epithelium. The control corneas remained the same throughout the first postoperative week except that the band of haziness and local corneal edema became slightly wider and more diffuse. By six or seven days the depression disappeared from the center of the narrow opaque band and the entire corneal surface was smooth, except at the points where the sutures entered the tissue. The band of haziness was also enlarged at these points to encompass an area approximately 0.5 mm. around the suture.

Fourteen to sixteen days after wounding the band of haziness was more restricted to the wound and shorter as the more peripheral ends of the incision became less obvious. The rest of the cornea was clear. By 21 days of healing the scar appeared less distinct and smaller, suggesting that wound contraction had begun.

Healing of Irradiated Corneas

In all of the eyes irradiated with doses of 10,000 rads of beta radiation, either prior to or at the time of surgery, the healing process was similar. Within two to three days after wounding, the central cornea was characterized by a diffuse haziness and edema, not confined to the wound perimeter as in the control corneas. Although epithelial cell migration recovered the incision, the wound began to gape open anteriorly before the end of seven days of healing. By nine or ten days the gaping was more severe, edema and opacity was more extensive, and some superficial degeneration of the cornea was noted along the wound edge and around the sutures. The anterior chamber was maintained only by the fibrin clot and the epithelial overgrowth. Some cases of neovascularization were noted as the perilimbal vessels began to extend into the cornea toward the nearest portion of the incision.

After 14 to 16 days of healing, the irradiated corneas gaped anteriorly some 1 to 2 mm., and superficial degeneration of the cornea around the sutures resulted in some of the sutures pulling loose from the tissue. Neovascularization was present in most cases, often

extending 2 mm. or more to the wound perimeter. Corneal opacity was so severe that the pupil was often obscure.

By the twentieth postoperative day, 85 percent of all irradiated corneas were characterized by gaping wounds, swollen stromal areas involving the wound and adjacent to the wound edge, severe corneal edema and corneal opacity in the central half, and neovascularization extending to the wound edge. More than 90 percent of all irradiated corneas had some anterior degeneration directly adjacent to the perimeter of the incision. This superficial degeneration, or "corneal melting," was apparently a manifestation of the lack of tissue regeneration at the wound, complicated by further degeneration along the perimeter of the wound and tension produced by the closing sutures.

Conjunctivitis and blephritis also accompanied the delayed healing of the cornea. The irradiated corneas were more prone to infection than the control corneas following termination of the topical antibiotic postoperative treatments. It is interesting to note that corneal edema is a relative measure of endothelial disfunction, and histological findings that the endothelium had not recovered the wound at this time exemplified this correlation.

Of the remaining eyes that were not quite so severe, all had retarded regeneration of the stromal connective tissue, some gaping of the wound, edema and swelling, haziness of the central cornea, and conjunctivitis, but not all had extensive neovascularization. In spite of some superficial degeneration, some retained the sutures in place which helped to maintain closure of the wound.

Gross examination and biomicroscopic observations indicated that the predominant characteristic of the delayed healing was the lack of connective tissue regeneration. The second most obvious problem was superficial degeneration and severe corneal edema which invited neovascularization from the perilimbal vessels. Even though the degree of inhibition varied somewhat from animal to animal, the difference between the non-irradiated control wound and the contralateral-irradiated wound was so obvious by the end of the first week that the code was not needed to identify the irradiated eye in a particular animal.

Some animals were followed for nine months after wounding. Healing was extremely slow and appeared to require at least three times longer for substantial connective tissue regeneration to occur. When stromal regeneration finally filled in the gaping wound the overall state of healing began to approximate normal healing characteristics found at one or two weeks after wounding. Edema and haziness persisted for several months, slowly decreasing until by eight or nine months the irradiated wounds appeared comparable to non-irradiated wounds of two or three months healing. Regression of the edema was the last symptom of delayed healing to disappear. Scar contraction had occurred and the residual remaining scar was as faint as controls and only about one-third the size of the original wound. Apparently, between six and nine months, the inhibited wound healing process began to achieve enough progress to insure eventual complete healing.

In contrast to the eyes that were irradiated either two and one-half months prior to wounding or at surgery, those eyes that had received 10,000 rads of beta radiation ten months prior to wounding did not exhibit as severe an inhibition and delay of normal healing. Apparently the lasting effect of the beta radiation, on the intact cells prior to injury, had begun to decrease during the ten month period between irradiation and wounding.

Throughout the entire ten month period between irradiation and surgery, the treated eyes were indistinguishable from the non-irradiated control eyes. No evidence of radiation damage was observed in any of the animals prior to the surgical wounding.

Six to seven days of healing were required to show any difference between the control wounds and the irradiated wounds in any animals treated ten months previously. At this time, however, more extensive haziness and edema were noted in the areas adjacent to the irradiated wounds. Gaping of the irradiated wounds was present, but not severe. By comparison to the other irradiated wounds it was apparent that some connective tissue regeneration had begun, although not as much as was noted as in the control eyes at a comparable healing time.

Throughout the second and third weeks the mild inhibition of healing in the irradiated corneas was characterized by edema and haziness of the central one-third of the cornea and some evidence of a partial lack of connective tissue regeneration in the wound.

Three of these animals were observed until nine weeks of healing. At this time the corneal haziness was confined to the immediate area of the wound, and wound shrinkage had begun; however, the wound edges gaped open slightly and remained swollen. Normal healing was still inhibited throughout the nine weeks. The animals were sacrificed at this time for histology and electron microscopic study.

It was apparent that, although the inhibitory effect of the prior irradiation treatment was wearing off, sufficient cellular damage remained to cause marked delay of normal corneal healing. Obviously, the beta radiation had affected more than just the epithelial cell layers as normal regeneration of the epithelial cells would have involved many complete turnover cycles. Thus, any epithelial cells present at the time of the irradiation would have been long since replaced before the cornea was wounded either two and one-half or ten months later. The characteristic lack of stromal connective tissue regeneration even in those animals irradiated ten months before wounding would suggest that the stromal keratocytes were affected by the beta radiation.

Tensile Strength of Corneal Wounds

Tensile strength measurements have been used as a measure of tissue repair for several years. Since the application of this method to corneal wounds (84), tensile strength measurements have been used as a simple tool to test the effect of many agents and circumstances

on the corneal repair process. This method has served as a measure of corneal collagen production and, therefore, repair of the corneal stroma.

Experiments designed to evaluate various surgical parameters that influence tensile strength of corneal wounds have established that the greatest rate of tensile strength development occurs in corneal wounds between one and four weeks of healing (71). During this time, tensile strength of the corneal wound increases from about 5 to 30 percent of the strength of the intact tissue. This period of maximal increase in wound tensile strength is preceeded by a lag period of six days, during which tensile strength is not detectable, and is followed by a period of several months wherein the increase of strength of the wound is very slow. More than three months is required to achieve a tensile strength of 50 percent of that of normal intact cornea.

The healing sequence of the cornea is apparently considerably slower than the same sequence in skin wounds, as the latter require only three weeks of healing time to regain the full tensile strength of intact skin (85).

Corneal tensile strength measurements have been used to test the effects of drugs such as topical steroids and idoxuridine on corneal wound healing (86). To date, this system has not been used to evaluate the effect of beta radiation on corneal healing.

Methods

Two groups of fifteen rabbits each were used to correlate the observed beta radiation induced delay in healing with the subnormal levels of tensile strength. One group received 10,000 rads of beta radiation immediately after surgical wounding, the other group was irradiated two and one-half months prior to injury. The limited number of animals that had been irradiated ten months before wounding precluded tensile strength measurements following wounding in this group.

All animals received identical, bilateral corneal wounds and postoperative treatment of atropine sulfate and Neosporin^{R} ophthalmic ointment.

A corneal strip 5 mm. wide was cut from each cornea, perpendicular to the wound, with a double-bladed, parallel razor blade knife. Sutures were removed and the strip was disected from the sclera at each end, leaving a narrow border of scleral tissue to aid in clamp fixation. The corneal strip was carefully fastened in special clamps and suspended on the tensiometer. A preweighed plastic bottle was hung from the corneal strip at the lower clamp and water was allowed to flow into the bottle at a constant rate. When the increasing weight was sufficient to break the wound, the bottle fell, stopping the addition of water automatically. The bottle and its contents were then weighed to determine the load required to break the wound.

Results

The tensile strength of the irradiated and non-irradiated corneal wounds, after 20 days of healing, are compared in Tables 1 and 2. The mean and standard deviation of the control wounds were within the ranges previously described by Gasset and Dohlman (71). In the group that had been irradiated immediately after surgery, the mean of the irradiated wounds was only one-third that of the control wound tensile strength mean. In those eyes that had received beta radiation two and one-half months prior to injury, the mean tensile strength of the irradiated wounds was only 23 percent of the mean tensile strength of the non-irradiated control wounds.

Paired statistics[1] showed a significant difference between the tensile strength of the control wounds and the contralateral wounds irradiated at the time of surgery, with 99 percent confidence ($p = 0.005$). Correspondingly, with 98 percent confidence, there was a significant difference between the tensile strengths of the non-irradiated and the irradiated corneal wounds in the group that were treated two and one-half months prior to injury ($p = 0.01$).

It should be noted that all of the non-irradiated control wounds demonstrated measureable tensile strength. Several wounds from both of the irradiated groups, however, pulled apart with just the weight of the 10 gm. clamps used to secure the corneal strip to the tensiometer.

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[1] Snedecor, G.W.: Statistical Methods Applied to Experiments in Agriculture and Biology, 5th ed., Iowa State University Press, Ames, Iowa, 1965, p. 49.

TABLE 1

TENSILE STRENGTH OF CORNEAL WOUNDS IRRADIATED AT SURGERY*

No. of Rabbits	Control Mean (non-irradiated) gm./5 mm.	Irradiated Mean gm./5 mm.	Paired Statistics** $t_{8:0.005}$	Paired Statistics** $t_{calc.}$
9	307	103	3.35	4.23

*Animals sacrificed 20 days post-op
**t-values calculated at 99% confidence

TABLE 2

TENSILE STRENGTH OF CORNEAL WOUNDS IN EYES IRRADIATED THREE MONTHS PRIOR TO SURGERY*

No. of Rabbits	Control Mean (non-irradiated) gm./5 mm.	Irradiated Mean gm./5 mm.	Paired Statistics** $t_{9:0.01}$	Paired Statistics** $t_{calc.}$
10	260	60	2.82	2.87

*Animals sacrificed 20 days post-op
**t-values calculated at 98% confidence

In contrast to the control wound tensile strengths at three weeks of healing, these several irradiated wounds were as weak as those normally found in the first six days, i.e., the lag period of corneal healing (71).

The tensile strength measurements were well correlated with the clinical observations of delayed corneal healing in irradiated wounds following surgery. After three weeks of healing the apparent lack of stromal connective tissue regeneration noted in the biomicroscopic evaluations were confirmed by the significant lack of tensile strength.

Histology

Six of the animals from each group were sacrificed, the eyes were enucleated, fixed in formalin, embedded in paraffin, and stained with hematoxylin and eosin.

Light microscopic examination showed striking differences between the irradiated wounds and the non-irradiated controls. Epithelial migration and overgrowth of the incision had occurred, as indicated by the biomicroscopic slit lamp inspections; however, the epithelial cells in the irradiated wounds were enlarged and abnormal in gross morphology even when compared to more peripheral epithelium in the same irradiated eye. The epithelium adjacent to and covering the incision was only three to four cells thick by the third week of healing. Invariably, some polymorphonuclear leukocytes (PMN) were present in the anterior stroma, aligned along the basal epithelial

cell layer. In contrast, the epithelial cells overlying the incision in the control eyes were eight to ten cell layers thick.

Notable in all the irradiated wounds was the complete absence of endothelial cells covering the posterior of the wound, and the lack of stromal tissue regeneration. Formation of the anterior chamber was achieved only through closure of the incision by the fibrin clot remaining in the wound. Fibroblastic proliferation was substantially less in the irradiated eyes than in the controls.

In control wounds, after three weeks of healing, sufficient stromal tissue regeneration had occurred to give an overall thickness at the wound that approximated the thickness of a normal cornea, and endothelium had completely covered the wound posteriorly.

A consistent observation in the irradiated wounds was a marked thinning of the stroma at the wound site, concomitant with severe swelling of the stroma directly adjacent to the wound edges. The actual stromal portion of the irradiated wounds were only about one-third the thickness of the control wounds.

Comment

Simple tensile strength measurements of healing corneal wounds have shown that doses of 10,000 rads of beta radiation cause a significant inhibition of normal repair. The lack of tensile strength development is well correlated with clinical and microscopic observations comparing the irradiated wounds with the non-irradiated controls. These radiation effects are equally demonstrable either when the beta

radiation is administered prior to, or immediately after, penetrating surgery. Paired sample statistics illustrate the high significance of these data when comparing the mean tensile strengths and standard deviations of the irradiated and the non-irradiated corneal wounds.

The observed lack of tensile strength in the irradiated corneal wounds appears to be a result of an inhibition of connective tissue replacement, perhaps a lack of collagen synthesis, and complications of normal corneal hydration that apparently result from the failure of the endothelium to close the wound posteriorly.

Epithelial migration occurs almost normally, even though the cells are enlarged and somewhat abnormal in morphology. Epithelial metabolism may be inhibited thereby inhibiting normal stromal regeneration and tensile strength development, however, this is not yet established.

The absence of endothelial migration and recovering the posterior wound would be expected to have a drastic effect on the normal stromal metabolism and maintenance of normal corneal hydration. The clinical observations and tensile strength measurements indicated that the abnormal edema and the lack of stromal regeneration might well be related to endothelial disfunction.

The inhibitory effect on corneal healing caused by beta radiation seems to be a long lasting one. Between two and one-half and ten months are required to begin recovery of normal corneal healing.

Even when ten months elapse between the irradiation and wounding, penetrating surgical wounds are characterized by a definite inhibition of connective tissue replacement, inhibition of fibroblastic proliferation, a lack of endothelial migration and a lack of normal deturgescence.

Figure 3. Control healing after nine days of healing. Note edema around incision.

Figure 4. Healing at nine days in cornea irradiated two and one half months before wounding. Wound is gaping severely. Note extensive edema and superior neovascularization.

Figure 5. Healing in non-irradiated eye at day 20. Note good scar formation and limited edema.

Figure 6. Healing in contralateral eye, irradiated two and one half months before surgery, at day 20. Severe wound gaping, neovascularization, and severe edema are visable. Anterior chamber is maintained only by fibrin clot in the wound.

Figure 7. Healing at day 20 in non-irradiated cornea (left eye). Note quiet conjunctiva, well defined scar indicative of good healing.

Figure 8. Corneal healing in contralateral eye (right eye of same animal as above) at day 20, following beta radiation immediately after surgery. Conjunctivitis, neovascularization, severe edema and anterior corneal melting characterize inhibition of healing.

#173 OS

#173 OD

Figure 9. Control eye healing at nine months. Wound contraction is notable and scar is disappearing.

Figure 10. Healing after nine months in contralateral eye that had been irradiated two and one-half months before wounding. Inhibition of healing has diminished during the nine-month healing period.

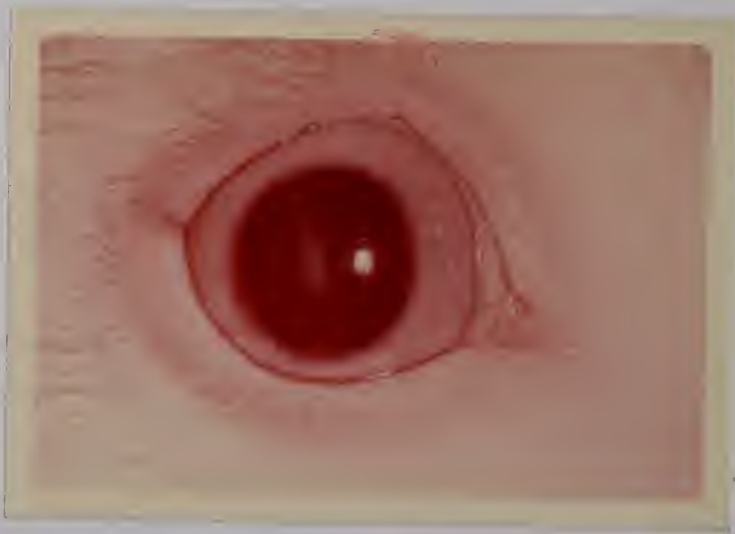


Figure 11. Healing in non-irradiated cornea at day 11.

Figure 12. Corneal wound after 11 days of healing, in cornea that recieved 10,000 rads of beta radiation 10 months prior to surgical wounding. Inhibition of healing is not as drastic. Edema is limited and some tissue regeneration is noted in the margins of the wound.

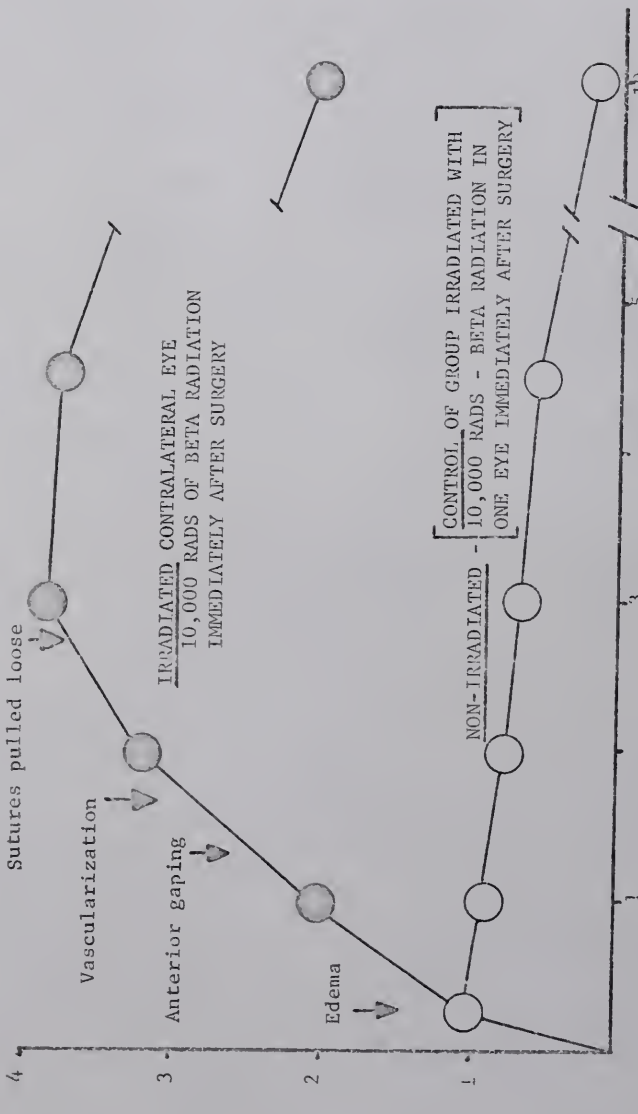
Figure 13. Healing at 21 days in same control eye as shown in Figure 11.

Figure 14. Healing in same irradiated eye as shown in Figure 12 after 21 days of repair. Edema and wound gaping have increased slightly since day 11 (see Figure 12). Inhibition of healing is considerably less than observed in corneas irradiated closer to wounding.

Figure 15. Control cornea after nine weeks of healing. Substantial wound contraction has occurred.

Figure 16. Contralateral eye, irradiated 10 months before wounding, shown at nine weeks after wounding. Although gaping is apparent some regeneration within the wound is noted. Persistant edema has attracted superior and inferior neovascularization.

Figure 17. Development of complications during inhibited healing. The severity of healing complications is expressed in terms of an empirical grading scale of 0 to 4+. Graph illustrates the typical difference found between irradiated and non-irradiated corneas in the same animal.


RELATIVE SEVERITY OF HEALING COMPLICATIONS
4
3
2
1
Sutures pulled loose
Vascularization
Anterior gaping
Edema
IRRADIATED CONTRALATERAL EYE
10,000 RADS OF BETA RADIATION
IMMEDIATELY AFTER SURGERY
NON-IRRADIATED - [CONTROL OF GROUP IRRADIATED WITH 10,000 RADS - BETA RADIATION IN ONE EYE IMMEDIATELY AFTER SURGERY]
1
3
5
10
HEALING TIME in Weeks

SECTION 4

CORNEAL ULTRASTRUCTURE DURING HEALING

Morphological transformations, cell migration, cell divisions, and changes in intercellular organization, as described by light microscopic studies, are only a part of the extensive changes that occur in normal corneal healing. The ultrastructural changes that occur within a particular cell during the repair process are related to the functional capability of the cell to meet the demands set forth by the cell's eventual role in the complex scheme of healing.

In normal corneal healing of surgical incisions, epithelial migration recovers the wound by 24 to 36 hours after injury. Extensive ultrastructural changes are noted in the epithelial cells during the period of lateral migration and for several months after the wound has been recovered by epithelium. At the wound site the basal cells of the epithelium lack a basement membrane and the hemidesmosomes which form the attachments between the basal cells and the basement membrane. Epithelial cells interdigitate and in the depth of the wound, the cells are joined by desmosomes. Epithelial mitochondria, in the intact cornea and in early corneal healing, appear as long, slender organelles with very sparce internal membranes and cristae. By 24 hours of healing, the epithelial mitochondria are

more numerous, larger, and possess a more complex internal structure (8). Frequently these mitochondria are found constricted at the center, and multilobed suggesting mitochondrial division and an increased metabolic activity. Even after two months of healing many alterations in epithelial ultrastrucutre still persist. The basement membrane is incomplete with large gaps that have not been filled in at the wound site (12). The basement membrane, where present, is often abnormally varied in thickness. Where large gaps exist, short segments of basement membrane, complete with basal desmosomes, can be seen within the gaps, suggesting that reconstruction of the basement membrane does not take place simultaneously among the basal epithelial cells that have recovered the wound.

The first stages of stromal healing are indexed by polymorphonuclear leukocyte invasion within 24 hours. Keratocytes are unchanged at this time and often mononucleated cells begin to appear near the wound. The mononucleated cells are characterized by short, pseudopod-like processes of the cytoplasm, yet they contain few endoplasmic reticula, mitochondria or ribonuclear protein (RNP) particles (8).

After 36 hours of healing, other mononucleated cells appear at the wound and keratocytes away from the wound undergo changes. These mononucleated cells contain extensive endoplasmic reticulum, larger and more numerous mitochondria, numerous RNP particles and long, thin cytoplasmic processes, typical of mature fibroblasts. In the

keratocytes away from the wound, rough endoplasmic reticulum appears as numerous free RNP particles and numerous vesicles.

Once the various cell types appear there is a migration and alignment within the wound. Presumably, collagen synthesis is initiated by the fibroblasts associated with the wound and the long term process of stromal reconstruction begins. Two months later, the fibroblasts are still present in the stroma, amid extensive collagen fibers with an abnormal variety of diameters. Collagen fibril orientation is random with occasional layering in the deeper stroma.

Corneal endothelium changes within 24 hours after corneal surgery. Nuclei become lobular, rough endoplasmic reticulum increases, and mitochondria are larger and more numerous. The endothelial changes are characteristic of an increase in metabolic activity of the cell and are consistent with the eventual regeneration of Descemets' membrane.

Descemets' membrane is thought to be secreted by the endothelium. It may be produced as the basement membrane of this cell layer (26). Regeneration occurs slowly after injury and after two months the new Descemets' membrane is still abnormally thin and irregularly layered.

The clinical observations, histology, and tensile strength measurements that were carried out earlier, all suggested that the lack of normal connective tissue regeneration and the absence of endothelial recovering of the posterior wound were a direct result of the beta radiation treatments. The radiation must also have caused

some alterations of the normal patterns of changes in ultrastructure that occur in corneal healing. It was hoped that any radiation-induced intracellular change might provide some index to the nature of radiation effects on normal cell function in corneal healing.

Methods

Immediately prior to enucleation, two drops of cold, 2 percent osmium tetroxide in phosphate buffer were placed on each cornea. The animals were sacrificed, the eyes enucleated and the cornea carefully dissected out with a 1 mm. rim of sclera attached. Separation of the cornea from the aqueous, iris, and lens capsule was carefully performed with forceps, taking care not to touch the anterior or posterior corneal surfaces near the control wounds.

The whole cornea was then fixed in cold 2 percent osmium tetroxide with phosphate buffer for two hours. The specimens were cut into small sections with a razor, dehydrated with alcohol, and embedded in Epon. Thick sections (1 to 2 μ) were stained with toluidine blue and inspected with the light microscope for electron microscopy orientation. E-M sections (500 Å) were cut with a diamond knife on a Porter-Blum microtome and stained with uranyl acetate and lead citrate. Specific collagen staining was carried out using phosphotungstic acid (PTA). Electron micrographs were taken with a Hitachi-II Type C electron microscope.

Corneal wound ultrastructure was examined after three weeks of healing to correlate with the observed lack of normal tensile strength of the wound following beta radiation. Electron microscopic studies were carried out on corneas that had received beta radiation immediately after surgery, or two and one-half or ten months prior to surgical wounding. Some specimens of corneas irradiated either two and one-half months or ten months before wounding were examined at nine weeks and nine months of healing.

Results

General Effects of Beta Radiation on Fine Structure of Healing Cornea

The effects of beta radiation on the intracellular changes that take place during corneal healing were essentially the same whether the radiation was administered immediately after or two and one-half months before wounding. If surgical wounding was delayed for ten months after beta irradiation, the magnitude of the radiation inhibition of healing was reduced and the ultrastructure of the involved cells was more normal.

In all of the irradiated corneas, if the wound adequately resisted infection during the period of radiation delay of normal healing, eventually the delay subsided and a normal healing pattern was established. The inhibition of normal healing was prominent at three and nine weeks of healing, fibroblasts were inhibited, and abnormal ultrastructure was noted. Collagen synthesis was drastically retarded and endothelium did not recover the posterior wound in those corneas irradiated either two and one-half months before or

immediately after wounding. By three weeks, in those corneas that received beta radiation ten months before wounding, the endothelium had spread to recover the posterior wound. After nine weeks, the fine structure of the involved fibroblasts appeared more normal, with a greater complement of intracellular organelles, and some new collagen synthesis was evident. Epithelial ultrastructure also appeared more normal.

After nine months of healing, those irradiated corneas that had remained free of infection had satisfactorily healed and appeared comparable to the non-irradiated, contralateral, wounded corneas. Light microscopic examinations showed only slight differences between the wounds in the irradiated and non-irradiated controls. Differences in fine structure were minimal, suggesting that the delay in healing was almost over. The details of these findings are described below.

Three Week Wounds

Ultrastructure of epithelium

Three weeks after surgery, non-irradiated corneal epithelium was characterized by some interdigitation, enlargement of intercellular spaces, a layering of some eight to ten cell layers deep, formation of some hemi-desmosomes, and the presence of portions of basement membrane where the epithelium attached to the stroma. The basal cell cytoplasm was esentially normal; however, clumps of ribosomal granules were scattered throughout, and tonofilaments were dispersed at random throughout the cytoplasm, with occasional bundling of tonofilaments in some scattered areas.

In contrast, the irradiated corneal epithelium was only one-fourth to one-fifth as thick as normal wounded epithelium. It consisted of two or three cell layers with some abnormally large cells near the wound edge. The epithelial cells stained much lighter with uranyl acetate-lead citrate than the non-irradiated cells, especially basal epithelial cells. Epithelial cells were interconnected by a few desmosomes and had distended intercellular spaces. There was a complete absence of any basement membrane along the stromal border, although many cytoplasmic processes were extended into the stroma.

The epithelial cytoplasm contained very few endoplasmic reticula, only scattered ribosomal granules, and tonofilaments that were found only in bundles. Mitochondria and other cellular organelles were poorly developed and few in number. No hemi-desmosomes connected the basal epithelial cells in the wound area. Basement membrane was found only in the basal epithelial layers peripheral from the wound area. Free RNP particles, Golgi apparatus, in lamellar form, and vesciles appeared normally distributed in the cytoplasm.

Ultrastructure of the stroma

Proliferative collagen fiber formation was evident in the control stromal wound areas immediately adjacent to the epithelial layers after the three week healing period. There was a large number of fibroblasts in the anterior stroma, but very few polymorphonuclear leukocytes.

The fibroblastic cells observed in these investigations were grouped into two different classifications based on distinct morphological differences. The first type, which will be henceforth referred

to simply as a true fibroblast, was characterized by extensive, distended endoplasmic reticula with extensive ribosomal granules lining the ER membrane, large numerous mitochondria, a well-developed Golgi complex, and increased flocculent filaments aggregated near the cell membrane. The second type, which shall be referred to as a fibroblast-like cell, was characterized by poorly developed cellular organelles, a small amount of endoplasmic reticulum, few ribosomal granules associated with the endoplasmic reticular membrane, few mitochondria, and yet an extensive appearance of intracellular flocculent filaments aggregated near the cell membrane, quite similar to the well-developed fibroblast.

The fibroblasts found in the anterior stromal areas of the control wounds were elongated, flattened, and adjoining each other, almost connected in an interlacing pattern, with collagen fibers filling the intercellular spaces. Some fibroblast-like cells were noted but more were apparent in the deeper portions of the wound.

In the central and posterior stromal areas of the control wounds collagen fibers were dispersed more randomly in a fibrin matrix amid scattered fibroblasts and fibroblast-like cells. Under higher magnification the collagen fibers were found to have a large variation in the diameter of separate fibers. Also, the collagen fibers in the posterior stroma of the wound were consistently found to be aggregated near the fibroblasts and fibroblast-like cells rather then scattered randomly throughout the intercellular spaces.

Irradiated stromal wound areas were characterized by very few cells at all in the anterior stroma, almost all of the fibroblast-like type, and no cells in the posterior stromal portion of the wound. The fibroblast-like cells that were present were not elongated nor flattened, but were roughly triangular in shape and quite different from the fibroblasts found in the control wounds. Collagen fibers were conspicuously absent from all areas of the wound. Only an occasional collagen fiber was found among the dense aggregates of fibrin that extended from the anterior chamber up to the epithelium. The wound matrix was composed of a low density cementing substance, and numerous fine filaments that were not banded and were occasionally found grouped in bundles. These bundles of fibrin filaments were often closely entwined and almost run together. This dense interweaving pattern became more compact as one scanned more posteriorly in the wound; however, collagen fibers were conspicuously absent even in deep layers.

Fine structure of endothelium

The control corneal endothelium had recovered the wound posteriorly very early in the three week healing period. The endothelium at three weeks was characterized by large intercellular spaces, which were rather straight passages between the cells rather than interdigitated. Terminal bars appeared at the posterior surface of the endothelial layer. The endothelial cell cytoplasm possessed normal Golgi apparatus and normal sized mitochondria; however, the cells were thickened in overall diameter. There was an increased amount

of endoplasmic reticulum that was notably distended, typical of some synthetic functioning. The cell membrane of the endothelial cells was undulated posteriorly along the anterior chamber. Although Decemet's membrane had not been reformed, there was a dense amorphous substance that had been formed along the anterior endothelial cell borders, suggesting that synthesis of the membrane was in progress.

The endothelium in the irradiated corneas had not recovered the posterior wound at this time. The endothelium peripheral to the wound area appeared normal all the way out to the limbus. Again, the anterior chamber was apparently maintained only by the epithelium anteriorly and the fibrin clot which closed the wound posteriorly.

All the radiation induced changes were equally demonstrable in three week wounds when the beta radiation was administered either immediately after or two and one-half months prior to wounding. In those corneas irradiated ten months before surgery, however, some differences were noted. By three weeks the endothelium had recovered the wound posteriorly, more fibroblasts were involved in the wound site, and some suggestion of connective tissue regeneration was recorded.

Nine-Week Wounds

In those corneas irradiated two and one-half months before wounding, anterior stromal degeneration was still evident after nine weeks of healing. In the corneas that received beta radiation ten

months prior to surgery, the inhibition of healing was considerably less. Apparently some recovery of the radiation damage had begun by ten months after irradiation.

Light microscopic examinations of the nine-week wounds in corneas irradiated ten months before wounding showed that endothelium covered the wound posteriorly and that some amorphous substance was being laid down adjacent to the endothelium. Stromal regeneration was noted in the anterior one-third of the wound and some fibrin had disappeared from the posterior portion of the wound.

Electron microscope studies showed that, although the epithelium was much less abnormal than in the corneas irradiated two and one-half months before surgery, some epithelial abnormalities were apparent. Superficial epithelial cells had less well developed and less numerous organelles. The mitochondria were small, simple, and few in number. The epithelial cytoplasm was still less electron dense than the cytoplasm found in the contralateral, non-irradiated cornea. Desmosomes were present at cell junctions, but intercellular spaces between the epithelial cells remained abnormally large.

Basal epithelial cells had a greater complement of mitochondria and other organelles than were found in the basal epithelial cells of the other irradiated groups. Basement membrane was still totally absent from the wound area, in contrast to the non-irradiated corneas, wherein segments of basement membrane were found along the basal epithelium covering the wound.

The anterior stroma of the irradiated corneas, at nine weeks of healing, showed a notable amount of newly synthesized collagen adjacent to numerous fibroblast-like cells and some few scattered fibroblasts. Most of the cells involved in the immediate wound area were similar to those previously described as fibroblast-like cells; however, the majority of these cells contained a substantial complement of smooth endoplasmic reticulum and more numerous, better defined, organelles than the fibroblast-like cells noted in the more severely inhibited corneas. These fibroblast-like cells noted at this time appeared more like inactive or non-synthesizing fibroblasts, rather than some morphologically different cell type or a primordial form of a developing fibroblast. A considerable quantity of fibrin remained scattered throughout the middle and posterior stromal portion of the wound. Cells were less numerous as one scanned posteriorly toward the endothelium. In the posterior of the stroma near the endothelium, more of the cells observed were identical to the fibroblast-like cells, lacking prominent organelles, found in the more damaged corneas which were irradiated at surgery.

Phosphotungstic acid (PTA) staining for collagen showed normal banded collagen fibers in the anterior stroma at nine weeks of healing. The concentration of collagen fibers was less in the corneas irradiated ten months before wounding than in the non-irradiated corneas.

Near the endothelial layer of the corneas irradiated ten months before wounding, sparse collagen fibers were found amid fibrin filaments. Along the endothelial cells an electron dense amorphous substance was found, almost adhering to and lining the endothelial

surface facing the stroma. This apparently was an initial stage in the regeneration of Descemet's membrane by the endothelium. The endothelium at nine weeks of healing was characterized by increased ribosomal-lined endoplasmic reticulum, increased mitochondria and vesicles, and enlarged intercellular spaces. The terminal bars at the endothelial surface junctions and the desmosomes were normal.

In summary, the ten month period between irradiation and wounding seemed to be sufficient to allow some recovery of the damage to the corneal cells and partial recovery of the normal repair capability.

Nine-Month Wounds

Corneal wounds after nine months of healing appeared clinically to be almost completely healed, with the exception of a superficial hazy scar, smaller than the original incision. Light microscopy shows that the basal cell epithelium in the wound area is not completely normal, and epithelium is thickened in the immediate area. Anterior stroma is somewhat disorganized and in the area of the scar the lamellar architecture has not been reestablished. The posterior half of the stroma has the lamellar collagen arrangement and appears normal except for greater than normal numbers of stromal connective tissue cells. Descemet's membrane has been reformed, the curled ends of the original, severed, Descemet's membrane are present, and the endothelium is normal. If it had not been for the presence of

severed ends of the original Descemet's membrane, it would have appeared that the wound had only been superficial and not penetrating.

After nine months of healing the irradiated wounds appear similar to the non-irradiated control wounds; however, the disorganized state of the stromal collagen arrangement is found at all depths of the stroma. The scar is much deeper, involving all of the stroma, and the stromal wound area has far fewer connective tissue cells than the non-irradiated. Descemet's membrane has been reformed and endothelium is intact.

Electron microscopic studies demonstrated that the epithelium at the wound was similar between the irradiated and the non-irradiated corneas. The stroma of the irradiated corneas was characterized by collagen bundles, organized in uneven lamellae-like layers, surrounded by mucopolysaccharide ground substance, and sparsely populated by normal keratocytes. Only rarely were fibroblast-like cells present and only a rare PMN or mononuclear leukocyte was found in the entire corneal wound.

Descemet's membrane, although thinner than normal, was completely reformed, but the surface of Descemet's membrane adjoining the stroma was not evenly nor clearly demarcated.

The endothelial layer of the irradiated corneas appeared normal. Endothelial cells had lost the increased endoplasmic reticulum found earlier in the delayed healing corneas. The mitochondria, Golgi complex, ER, and other cellular organelles appeared normal. Tight junctions between cells formed normal compact, intercellular spaces

ending with normal terminal bars at the anterior chamber side of the cell layer. The endothelium appeared viable and functioning, based on its complement of internal structures within each cell and the normal interdigitation of adjacent cells.

Comment

Transmission electron microscopic studies have shown that changes in the fine structure of epithelial cells, fibroblastic cells, and endothelial cells are part of the beta radiation inhibition of normal corneal repair. Poorly developed intracellular organelles and a conspicuous lack of collagen synthesis were consistent with an apparent decrease in cellular activity and the lack of tensile strength found in the preceeding experiment.

The most prominent radiation induced changes observed in the basal epithelial cells that recover the wound were: (1) few and poorly developed mitochondria, endoplasmic reticulum (ER), and other cellular organelles, (2) a notable lack of hemi-desmosomes and enlarged intercellular spaces, and (3) a lack of basement membrane regeneration.

Fibroblasts and fibroblast-like cells were found in the normal healing stroma; however, fewer cells were found in the anterior portions of the wound and no cells were found in the posterior wound. In the irradiated corneas, among what few cells were involved in the wound, fewer fibroblasts and a higher proportion of fibroblast-like cells

contrasted with the high ratio of fibroblasts to fibroblast-like cells found in the non-irradiated corneas. The fibroblast-like cells lacked well-developed cellular organelles, and distended, ribosomal granule-lined endoplasmic reticulum found normally in synthesizing fibroblasts as described by Ross (87). In addition, the fibroblast-like cells were not flattened, elongated, nor functionally arranged (in almost interconnecting fashion) as were mature fibroblasts found in the normal heaing corneal wounds. Whether these two types of fibroblastic cells are intermediate forms of developing fibroblasts, as described previously in healing corneas (88), or whether they are functionally different fibroblasts as found in tendon regeneration (89) is not well understood.

The beta radiation treatments apparently prohibited the appearance of mature, synthesizing, fibroblasts in the stromal areas of the wound. Collagen synthesis was also inhibited by the radiation, either as a result of the fibroblast inhibition, or concomitant with the lack of mature fibroblasts normally found in the healing cornea.

It has been reported earlier (90) that the most distinctive intracellular feature of the mature fibroblast, i.e., extensive, highly-developed rough endoplasmic reticulum (ER), is the organelle commonly associated with extracellular protein synthesis, and presumably responsible for collagen synthesis. The most obvious difference between the fine structure of the fibroblast-like cells and the mature fibroblasts was the absence of extensive rough endoplasmic

reticulum in the former cell. In the irradiated corneas where the proportion of fibroblast-like cells made up almost the entire population of cells involved in the wound, collagen synthesis was conspicuously absent even after three weeks of healing. Only after nine weeks, when the proportion of mature fibroblasts had increased, was some minimal collagen synthesis observed in the corneas that had been allowed the longest interval between irradiation and surgery.

The presence of the mature fibroblast seemed necessary for collagen synthesis in the healing cornea. This observation is consistent with the current concepts of the functional capability of the fibroblast and synthesis of collagen precursors with fibroblasts (90, 91).

The fibroblast-like cells that well out-numbered the mature fibroblasts in the irradiated corneas were morphologically, and probably functionally, different from connective tissue cells capable of collagen synthesis. This observation was further supported by the conspicuous absence of any tropocollagen or other pre-collagen macromolecular aggregates near the fibroblast-like cells. If the cells were capable of intracellular synthesis of collagen precursors, which is dubious in the absence of extensive rough ER, some indication of these macromolecules should have been demonstrable either within the cell's Golgi, or cytoplasm, or extracellularly adjoining the cell. Therefore, the lack of collagen synthesis in the irradiated corneal wounds would seem to be correlated with the lack of cells that are capable of synthesis, rather than some inter-

ference with the extracellular aggregation of tropocollagen into the classical, banded collagen fibrils.

The radiation inhibition of endothelial migration emphasized the difference between the relative radiosensitivities of the epithelial and endothelial cell layers. Epithelial cells received higher doses of beta radiation than did endothelial cells, yet, epithelial migration was not inhibited. This observation is inconsistent with classical guidelines of radiosensitivity of different cells based on their degree of differentiation and frequency of cell division (92). Apparently, radiation damage to the ability of a cell to migrate and the overall radiosensitivity of the cell are two separate parameters of radiation effects on cells.

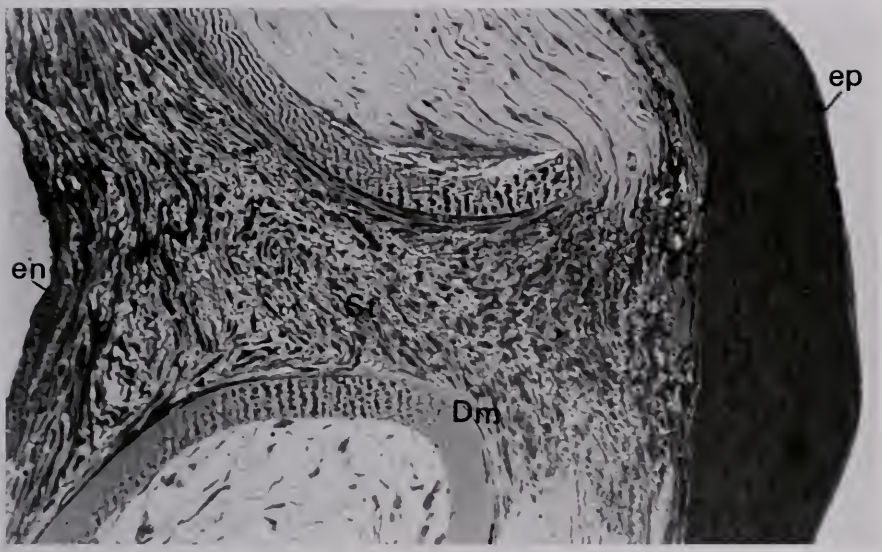
It was interesting that the intracellular characteristics of the intact peripheral endothelium in the irradiated corneas at three weeks of healing, seemed normal even when the endothelium did not spread and cover the wound posteriorly. When the radiation inhibition decreased and endothelial migration was observed, the fine structure of the endothelial cells was characterized by increased organelles and particularly rough ER, normally associated with increased protein synthesis. Increased protein synthesis would be expected in an expanding, spreading cell. At later stages of healing the intracellular fine structure of endothelial cells showed only minimal amounts of ER, indicating that the need for increased protein synthesis had diminished. Although current concepts would suggest that endothelial protein synthesis was still needed for

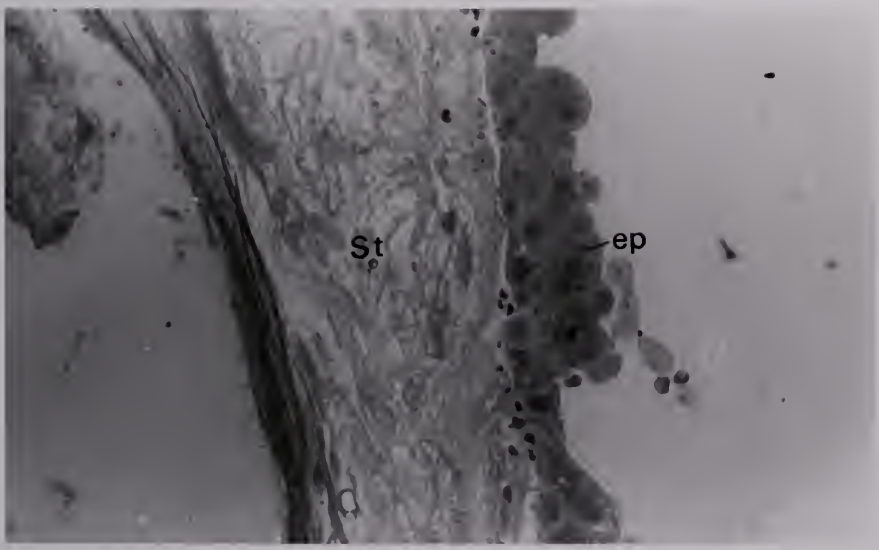
secretion of Descemet's membrane, it seems reasonable that once the total demands for protein (needed for both cytoplasmic expansion and extracellular products) decreased, by the amount required for cell expansion, the need for such an extensive rough ER would likewise decrease. Hence, the disappearance of extensive rough ER following cessation of endothelial migration was not totally unexpected.

It was hoped that the succeeding experiments, which were designed to examine the enzyme activity and nucleic acid synthesis in both normal and irradiated corneal wounds, would give a better understanding of the importance and interrelationships of the ultrastructural differences noted in the irradiated, wounded corneas.

Figure 18. Histological section of control corneal wound at three weeks of healing. Showing: thickness of regenerated wound, epithelium (ep), severed Descemet's membrane (Dm), fibroblasts in stroma (St), and endothelium (en) recovering the posterior wound. Epon, toluidine blue, x200.

Figure 19. Section of wound in contralateral irradiated eye, at day 21. Demonstrating: abnormal, enlarged epithelial cells (ep), lack of stromal regeneration (St), and absence of endothelium. Epon, toluidine blue, x200.


ep
en
St
Dm


St
ep

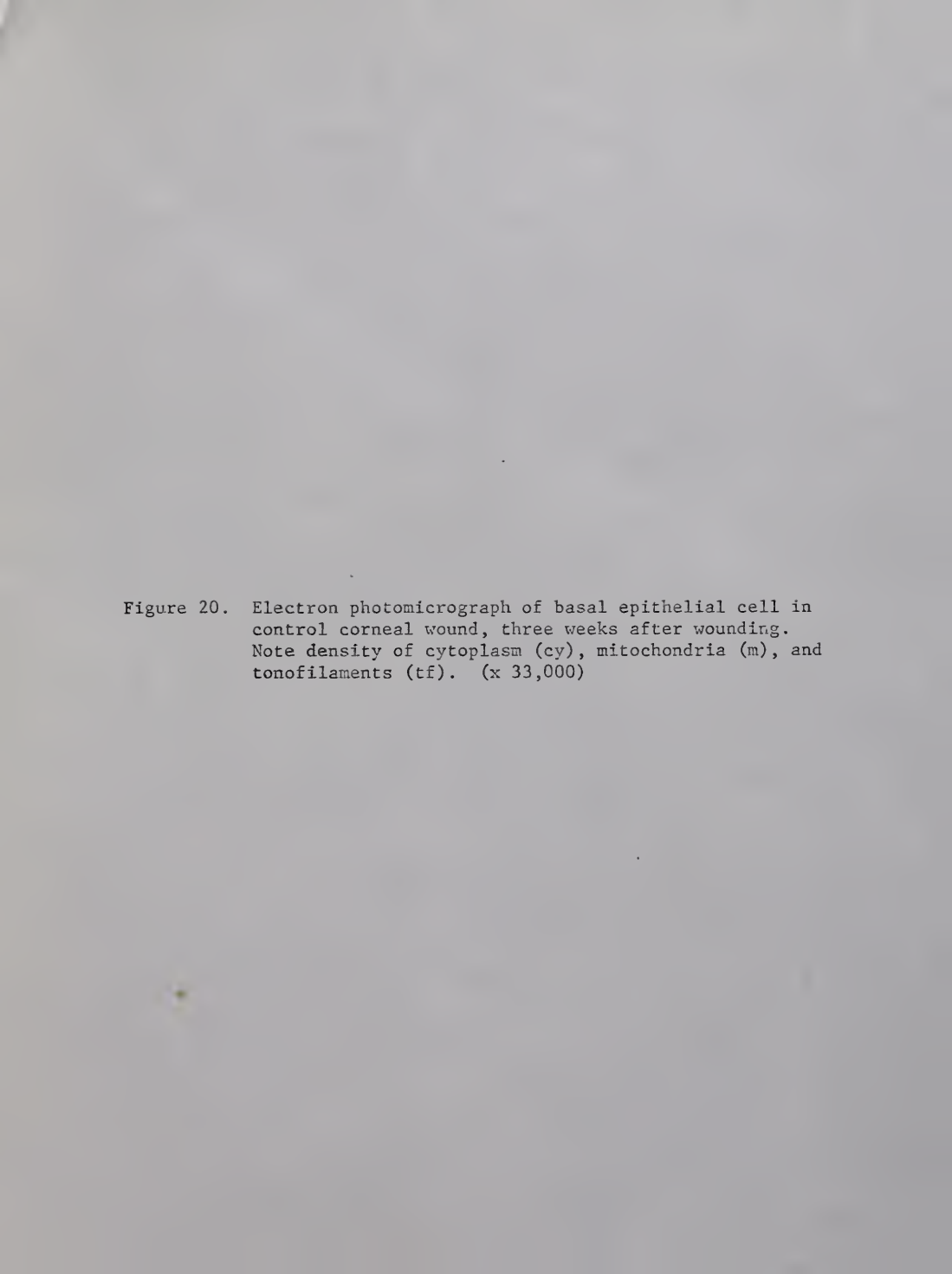


Figure 20. Electron photomicrograph of basal epithelial cell in control corneal wound, three weeks after wounding. Note density of cytoplasm (cy), mitochondria (m), and tonofilaments (tf). (x 33,000)

m
CM

Figure 21. Electron photomicrograph of basal epithelial cell in irradiated corneal wound, three weeks after wounding. Note less dense cytoplasm (cy), fewer, poorly developed mitochondria (m), vesicles (v), and bundled filaments (tf). (x 25,000)

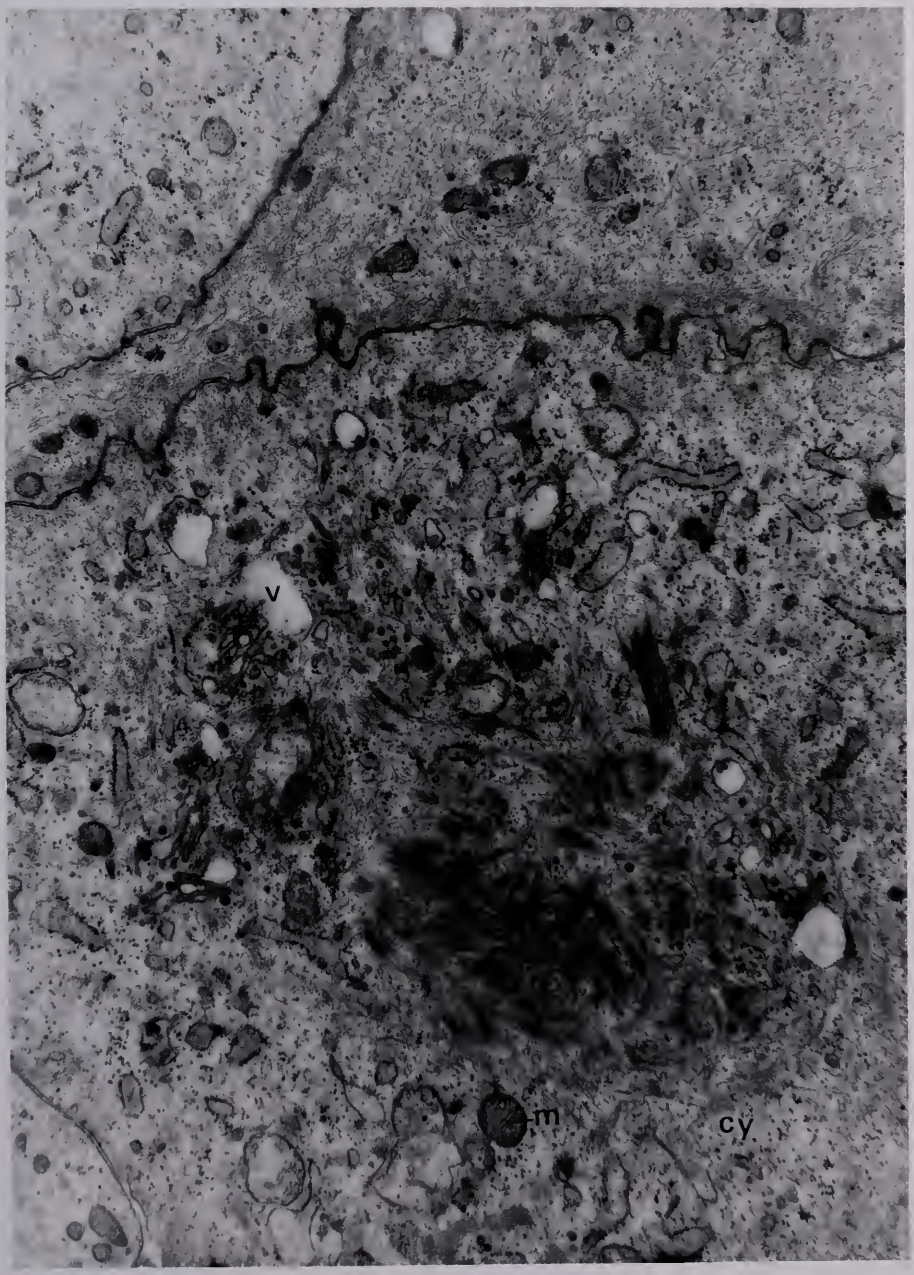

v
m
cy

Figure 22. Electron photomicrograph of basal epithelium cell near periphery of wound in irradiated cornea at three weeks of healing. Showing: polymorphonuclear leukocyte (pmn) interposed between basal epithelial cells and severed basement membrane (bm). Note poor cytoplasmic density and few cellular organelles in epithelial cells. (x 5,000)

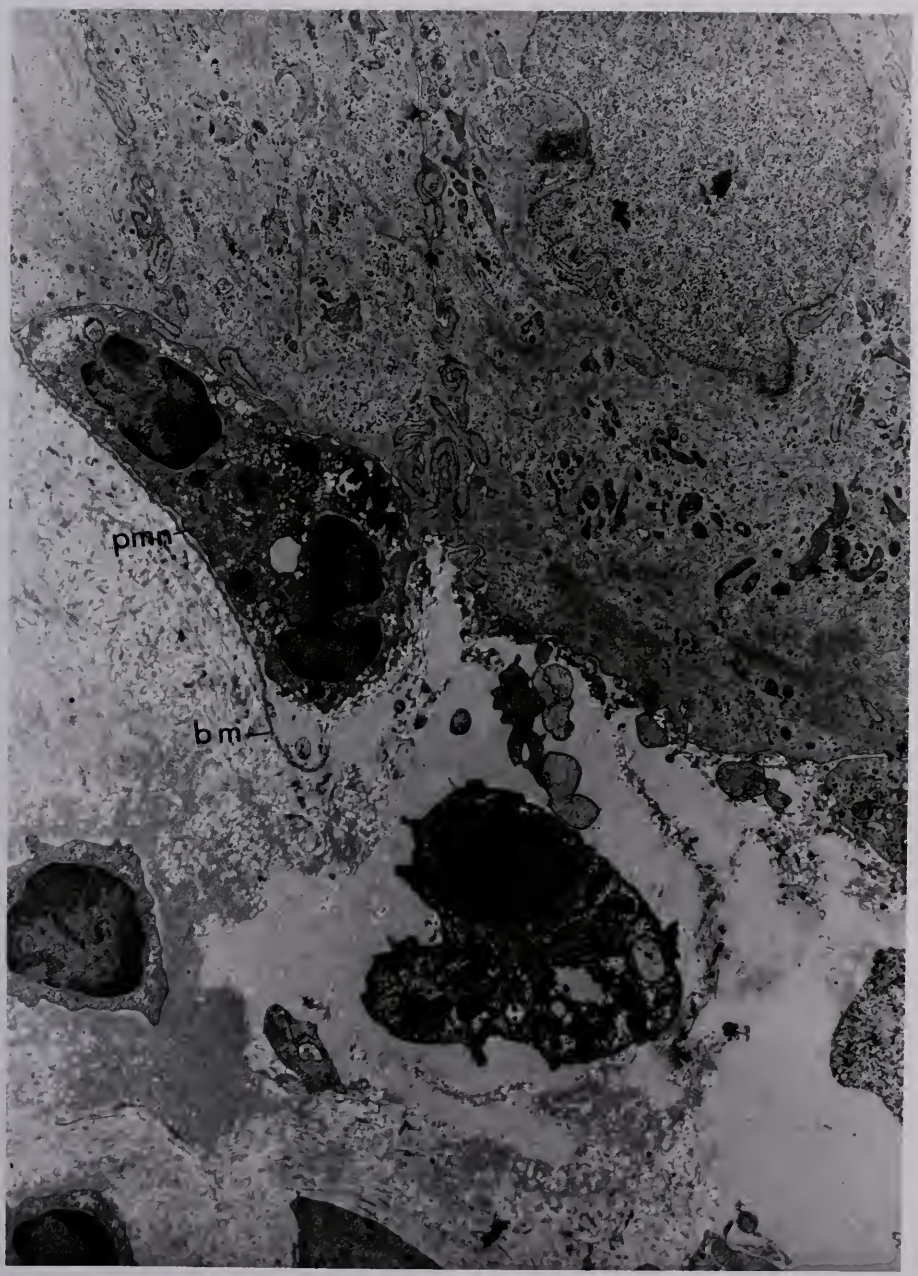

pmn
bm

Figure 23. Electron photomicrograph of basal epithelial cell in center of irradiated corneal wound. At three weeks of healing, note absence of basement membrane along epithelial cells (ep), polymorphonuclear leukocytes (pmn), and lack of fibroblasts. (x 7,500)

ep
pmn

Figure 24. Electron photomicrograph of stromal fibroblastic cells in anterior stroma of corneal wound. Showing both fibroblasts (Fb) and fibroblast-like cells (Fbl). Note the extensive mitochondria (m), Golgi apparatus (G), and rough endoplasmic reticula (rer) in the fibroblasts. Also note the fine filaments (ff) aggregated inside the cell membrane of both cell types and the few cellular organelles of the fibroblast-like cells, particularly the lack of rough ER. (x 15,000)

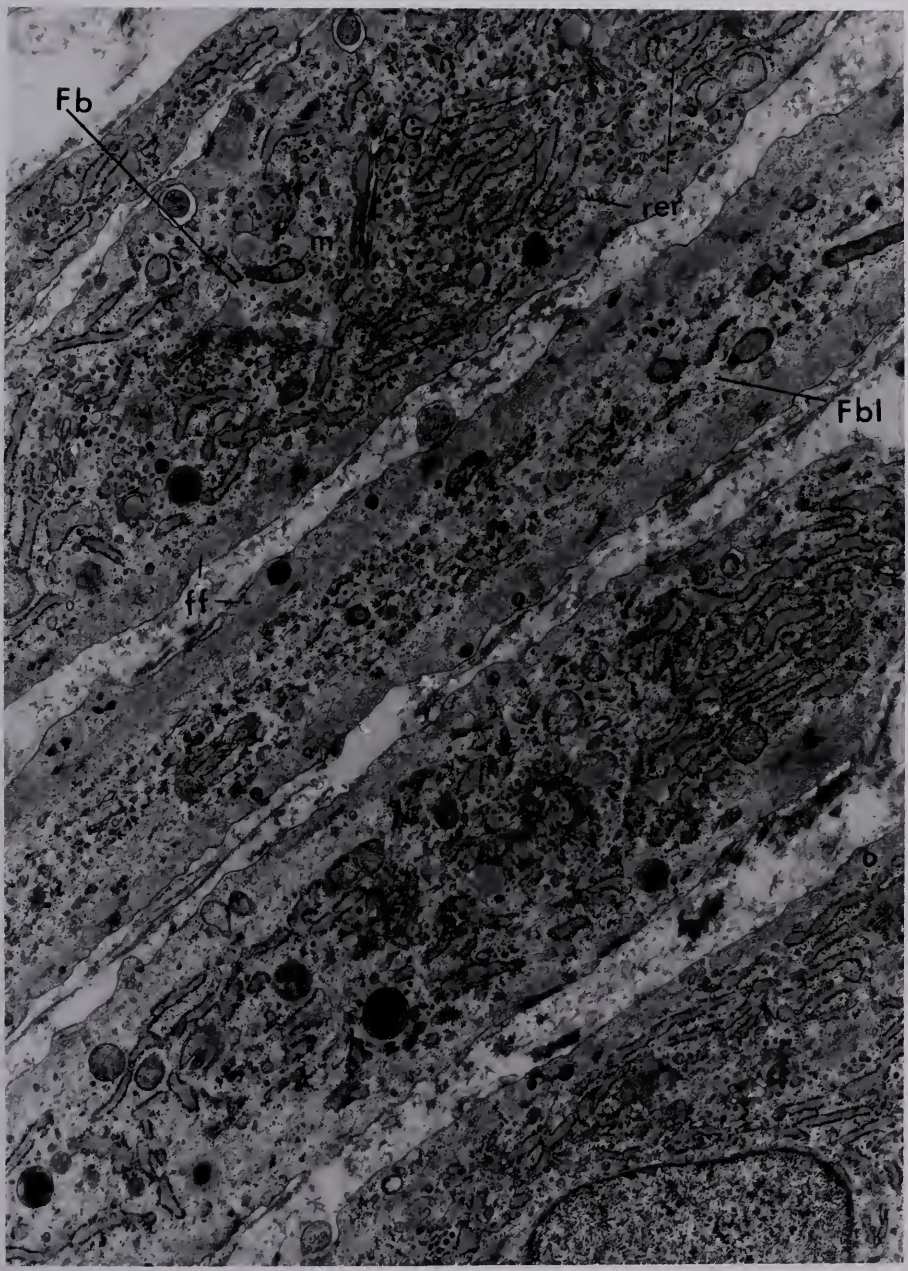

Fb
G
m
ret
Fbl
ff
b

Figure 25. Electron photomicrograph of fibroblast-like cells in irradiated corneal wound. Note fibroblast (Fb), keratocyte (Kr), and fibroblast-like cells (Fbl). (x 12,500)

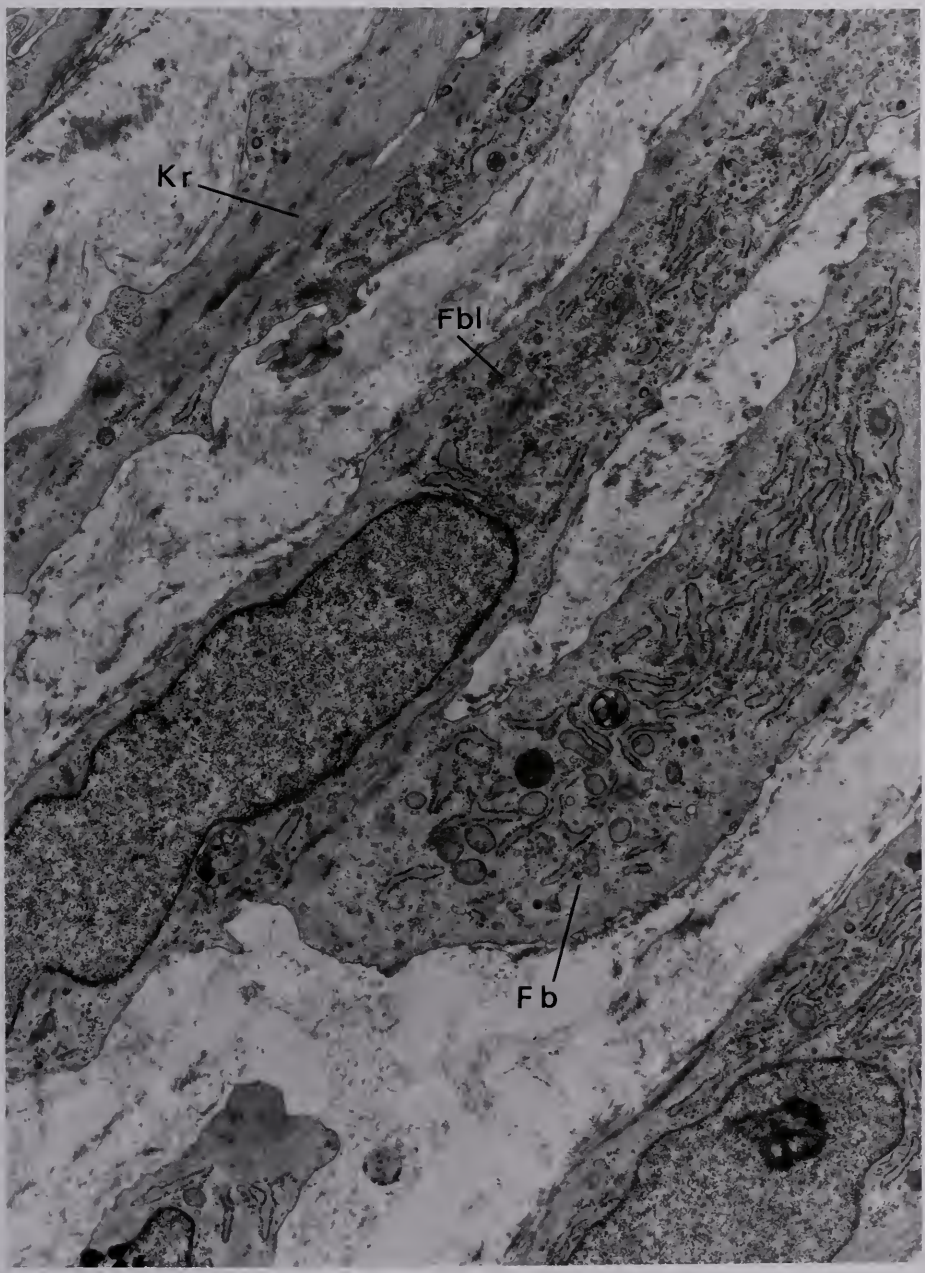

Kr
Fbl
Fb

Figure 26. Electron photomicrograph of fibroblast-like cell (Fbl) in anterior stroma of non-irradiated corneal wound. At three weeks of healing, note lack of rough ER, no extracellular collagen, yet extensive Golgi apparatus (G). (x 5,000)

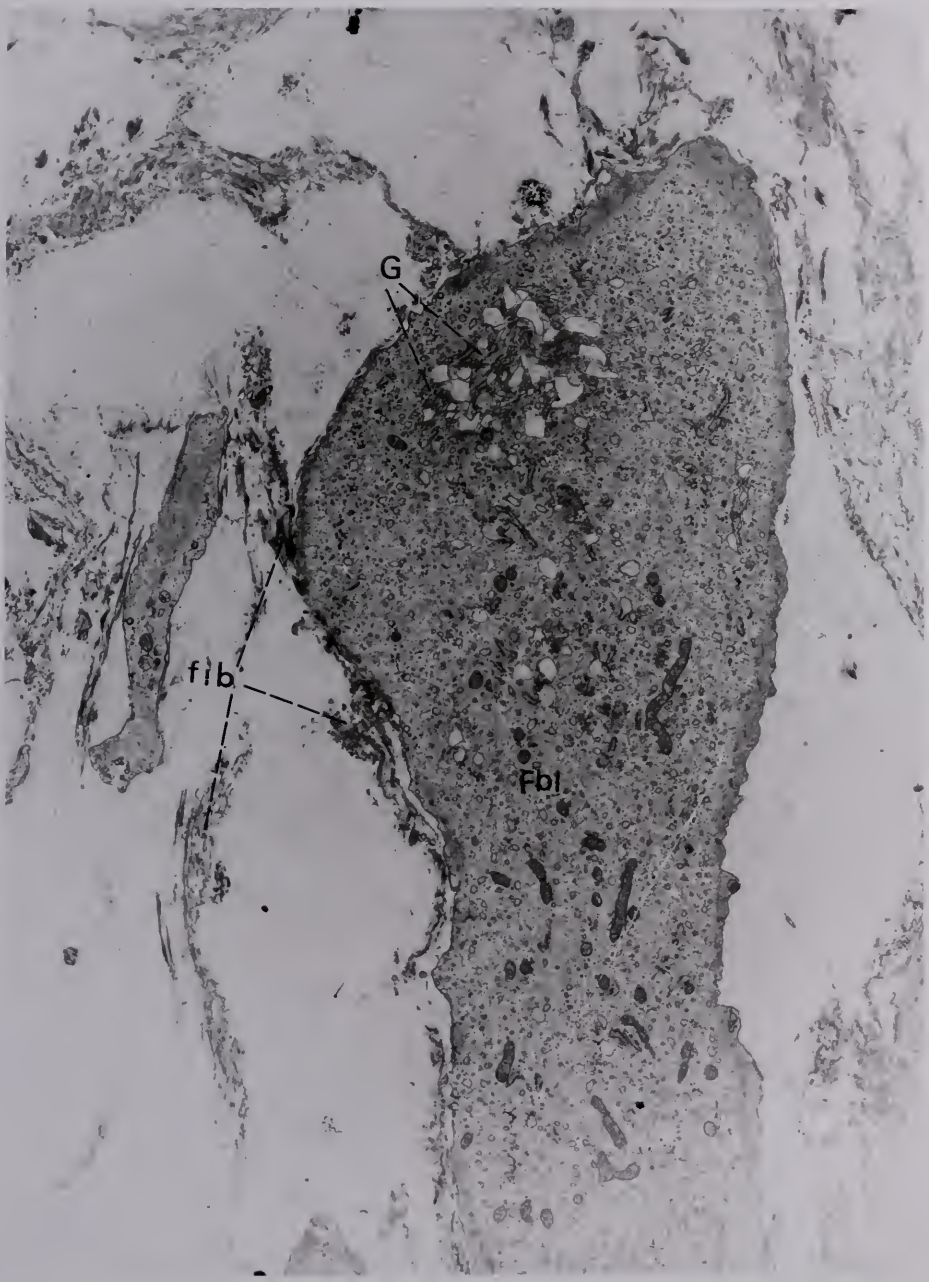

G
flb
Fbl

Figure 27. Electron photomicrograph of fibroblast in posterior stroma amid collagen. Note extensive rough endoplasmic reticula (rer), Golgi complex (G), and extensive collagen fibers (coll). (x 17,500)

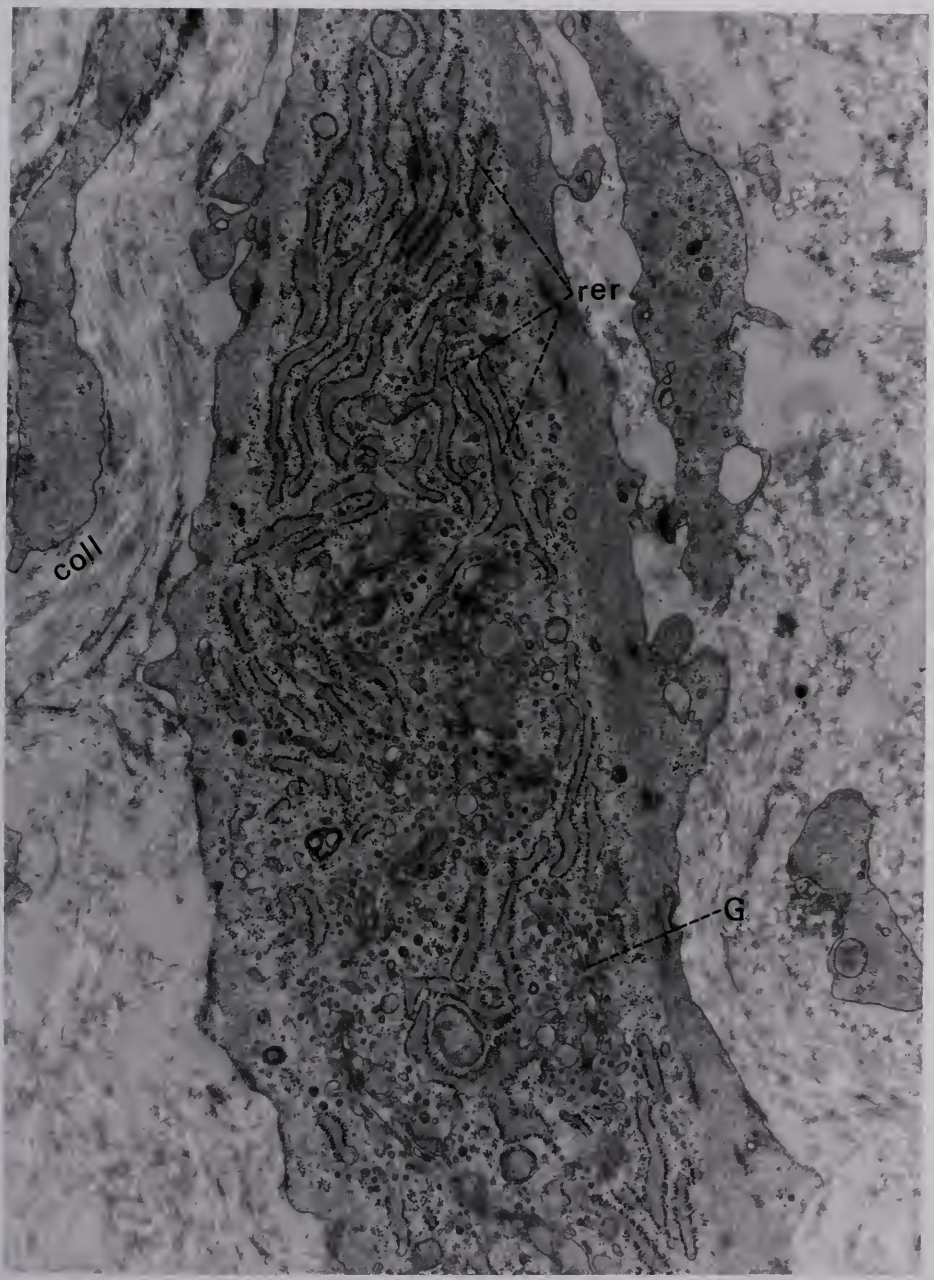

rer
coll
G

Figure 28. Electron photomicrograph of middle stroma of irradiated corneas at three weeks. Cell in center of picture is fibroblast-like cell. Note absence of collagen around this cell, but many fibrin filaments (fib). (x 5,000)

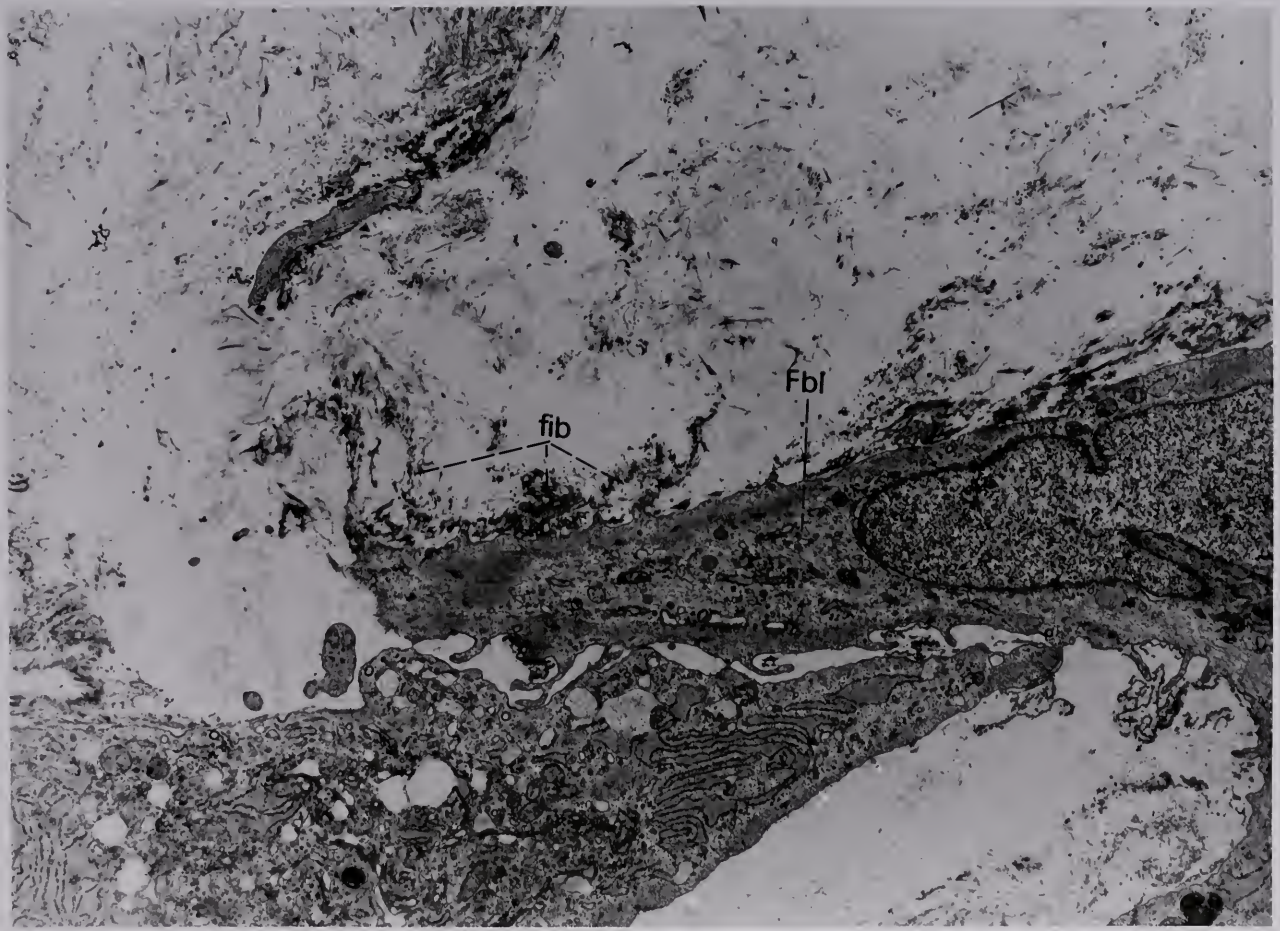

fib
Fbl

Figure 29. Electron photomicrograph of newly synthesized collagen at three weeks of healing (PTA stain). Note variety of diameters of newly synthesized collagen seen in cross-section. (x 50,000)

coll
xs

Figure 30. Electron photomicrograph of central wound - only isolated collagen fibril amid fibrin filaments. Lack of collagen filaments (cf) is characteristic of irradiated corneas following wounding. Compare with Figure 29, collagen synthesis in controlled cornea at this same time. (x 50,000)

rib
cf

Figure 31. Electron photomicrograph of endothelium in control cornea at three weeks. Endothelium (en) cells are joined by terminal bar (tb), Descemet's membrane (Dm) is being reformed as shown between endothelium and stromal fibroblast (Fb), amid newly synthesized collagen (coll). (x 15,000)

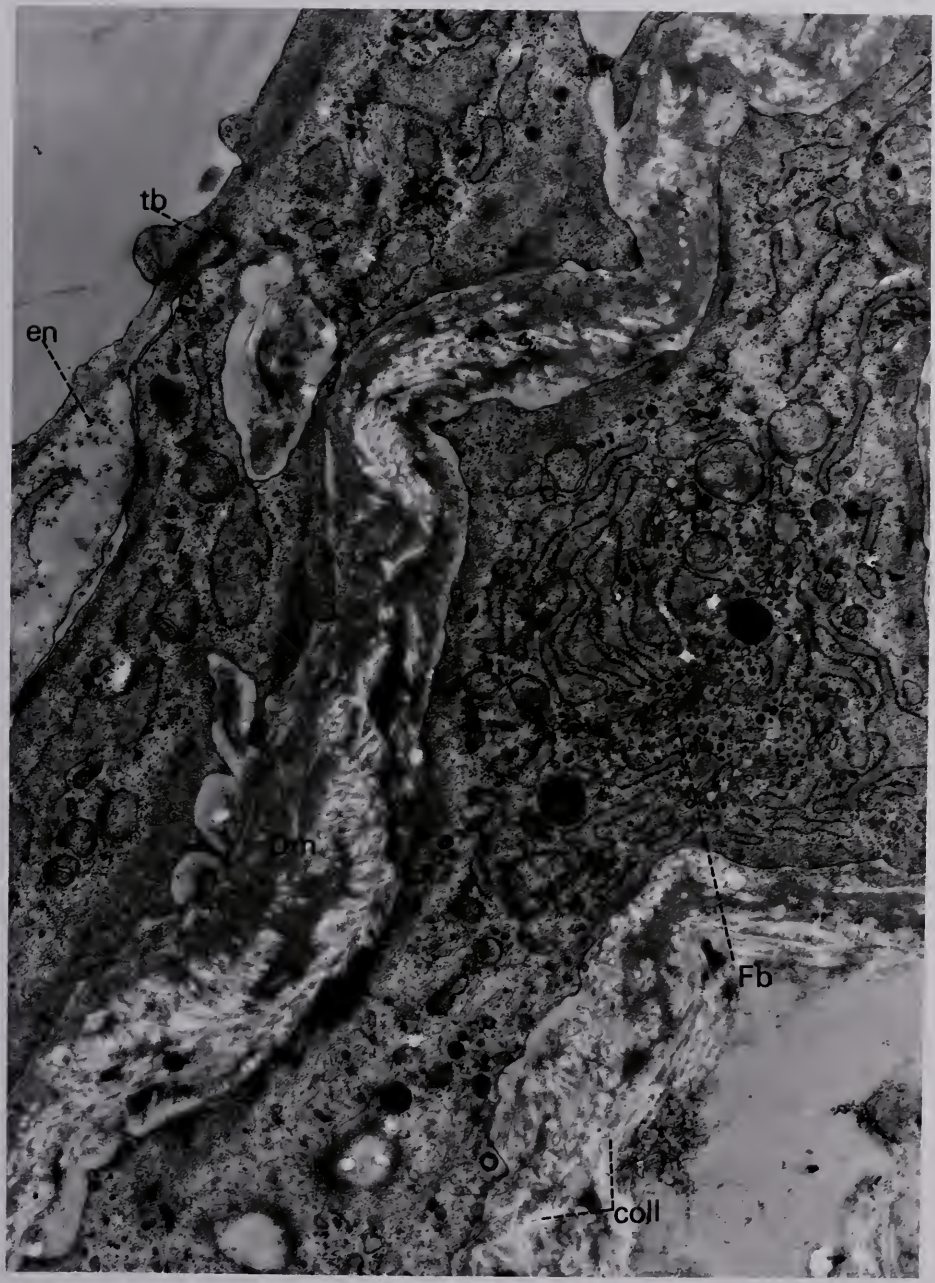

tb
en
Fb
coll

Figure 32. Electron photomicrograph of posterior stroma at three weeks of healing showing fibroblast amid collagen. In non-irradiated cornea collagen (coll) synthesis is extensive as illustrated here in posterior stromal wound. Note lymphocyte (Lym) and typical fibroblast (Fb) containing well developed cellular organelles. (x 7,500)

Lym
coll
m
Fb

Figure 33. Electron photomicrograph of detailed fine structure of endothelium. Non-irradiated endothelium contains elaborate Golgi appartus (G) note Descemet's membrane (Dm) being formed amid many collagen fibrils (coll). (x 20,000)

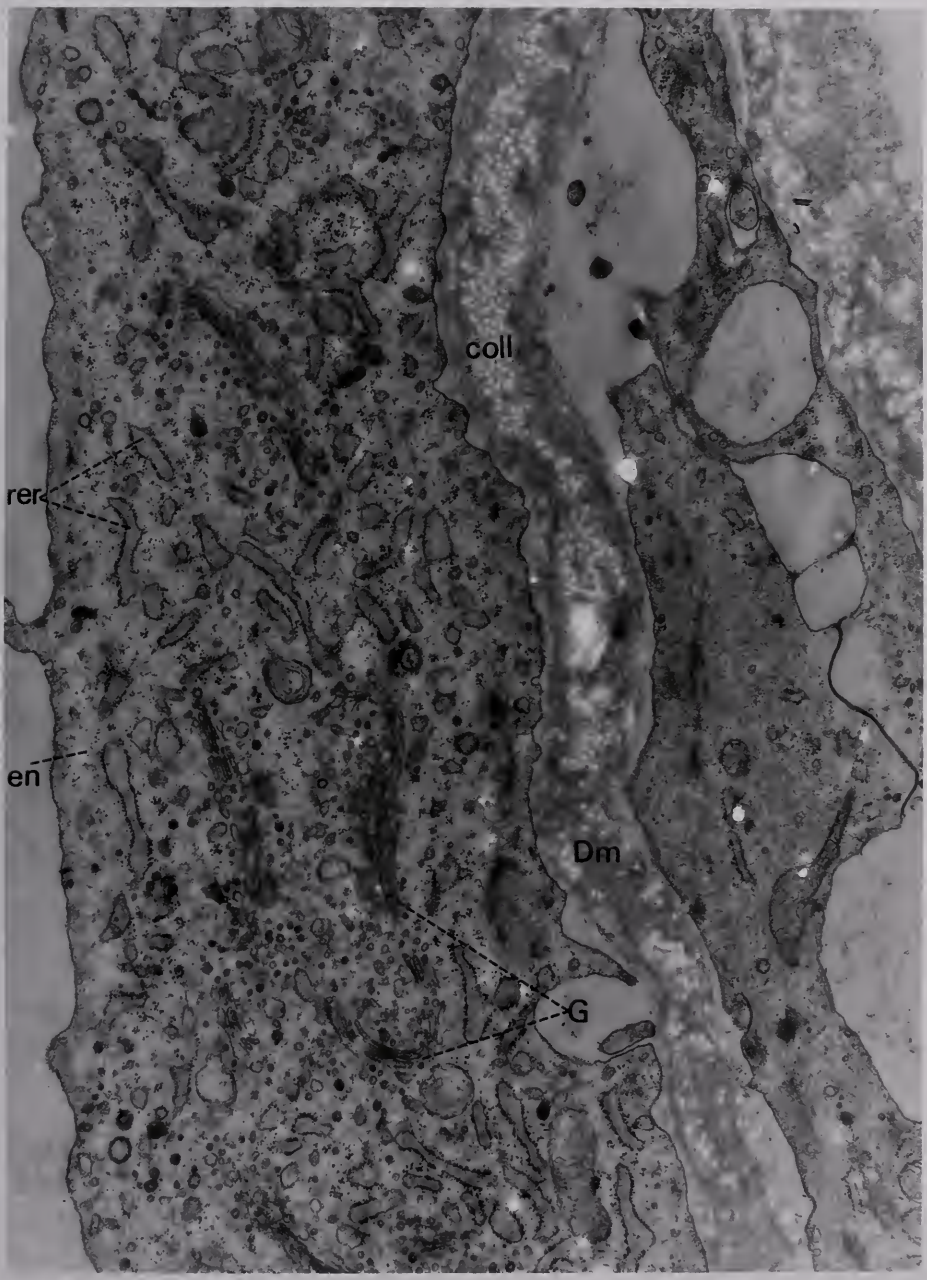

coll
rer
en
Dm
G

Figure 34. Electron photomicrograph of endothelial cell junction at three weeks of healing in normal cornea. Higher magnification of control endothelium shows terminal bar (tb) and intracellular spaces bridged by desmosomes. Extensive rough ER (rer) is indicative of protein synthesis by endothelial cells. (25,000)

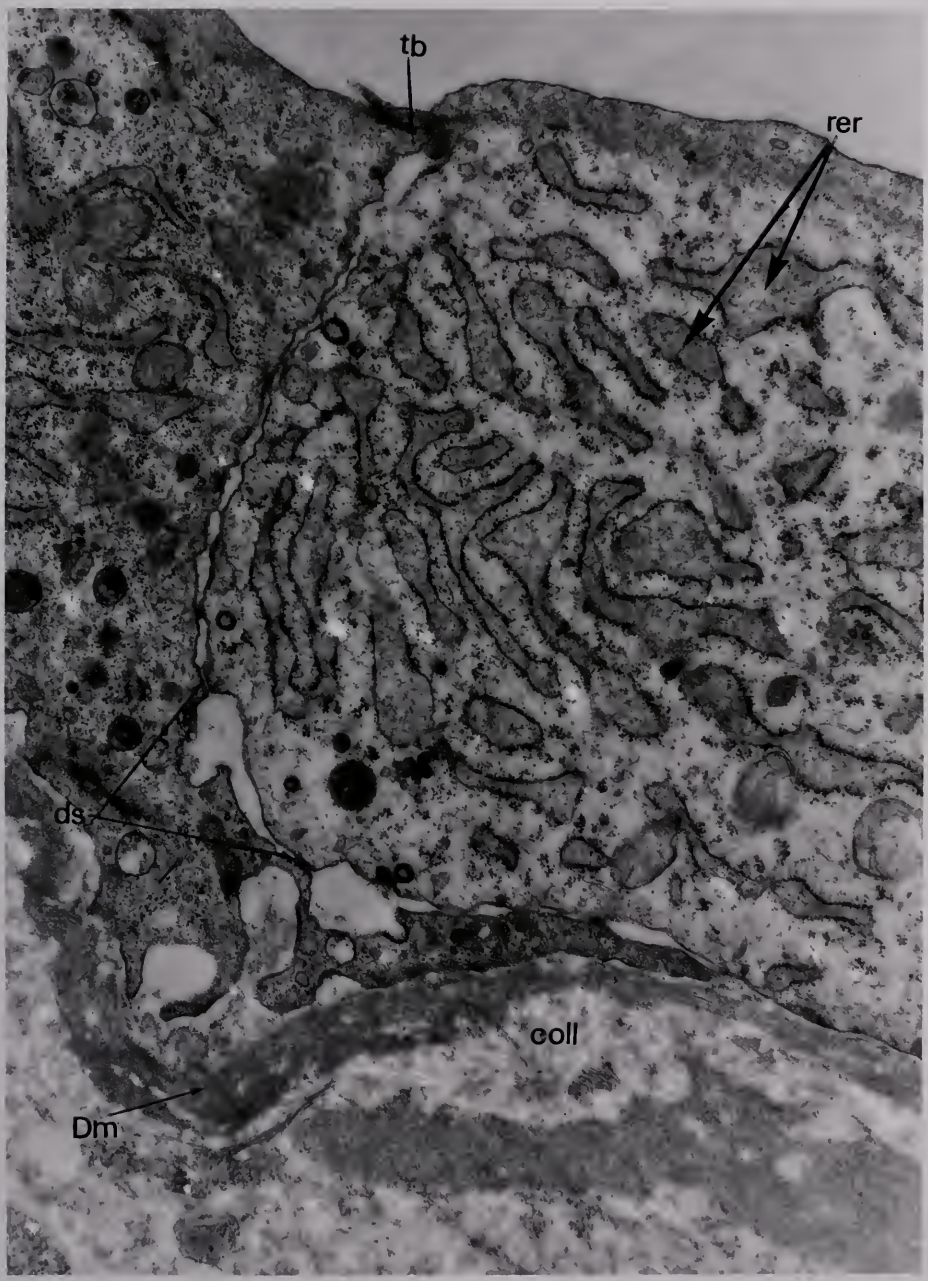

tb
rer
ds
coll
Dm

Figure 35. Electron photomicrograph of fibrin filament detail in irradiated corneal wound at three weeks of healing. Higher magnification of posterior stroma in irradiated cornea after wounding shows bundles of fibrin (fib) but no collagen filaments whatsoever. (x 50,000)

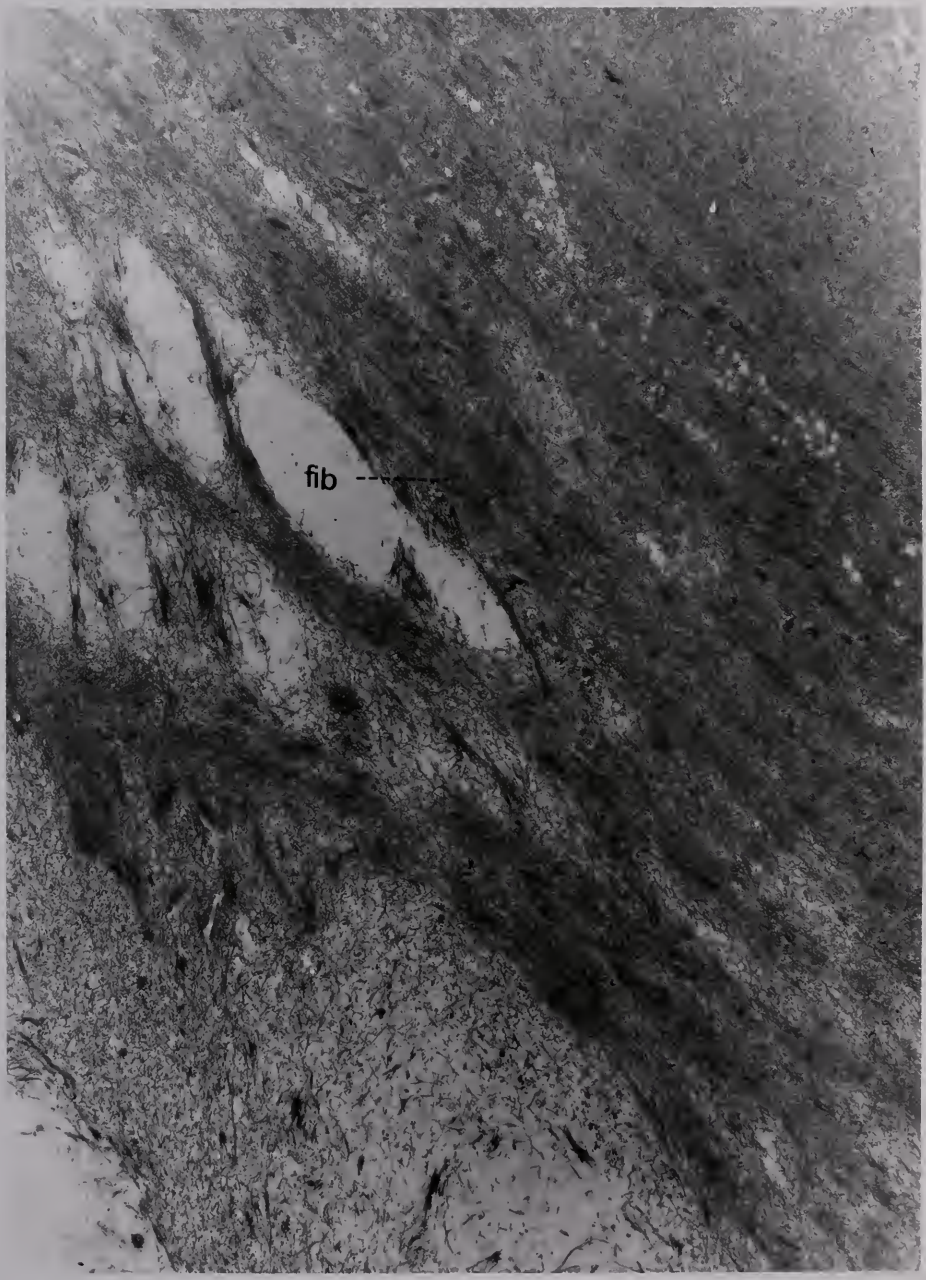
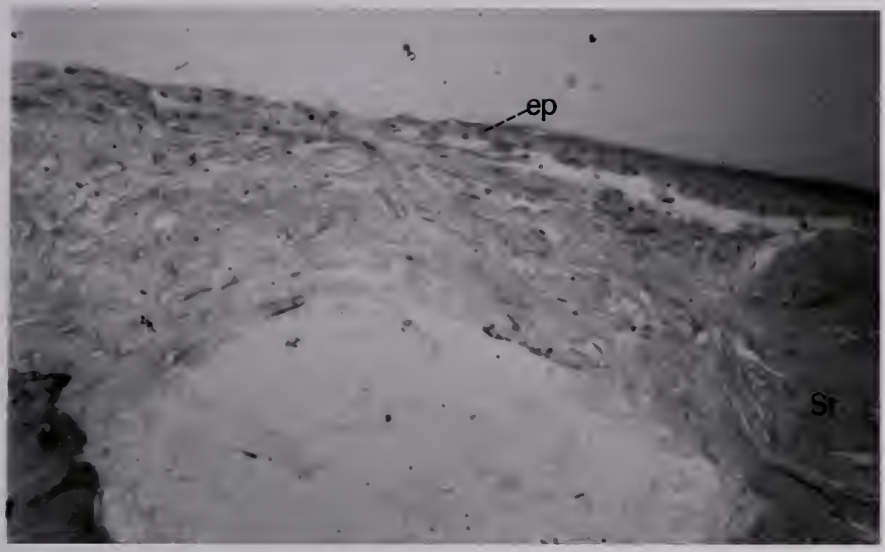

fib

Figure 36. Electron photomicrograph of fibrin filament detail in irradiated corneal wound at three weeks of healing. Higher magnification of posterior stroma in irradiated cornea after wounding shows bundles of fibrin (fib) but no collagen filaments whatsoever. (x 50,000)

fib

Figure 37. Photomicrograph of anterior corneal wound after nine weeks of healing in cornea irradiated 10 months before wounding. By this time anterior stromal regeneration is well underway. (x 250)

Figure 38. Photomicrograph of posterior irradiated wound after nine weeks of healing. Posterior stroma has not regenerated however, endothelium (en) has recovered wound and has begun secreting new Descemet's membrane (Dm). (x 250, same specimen as Figure 37)


ep
St

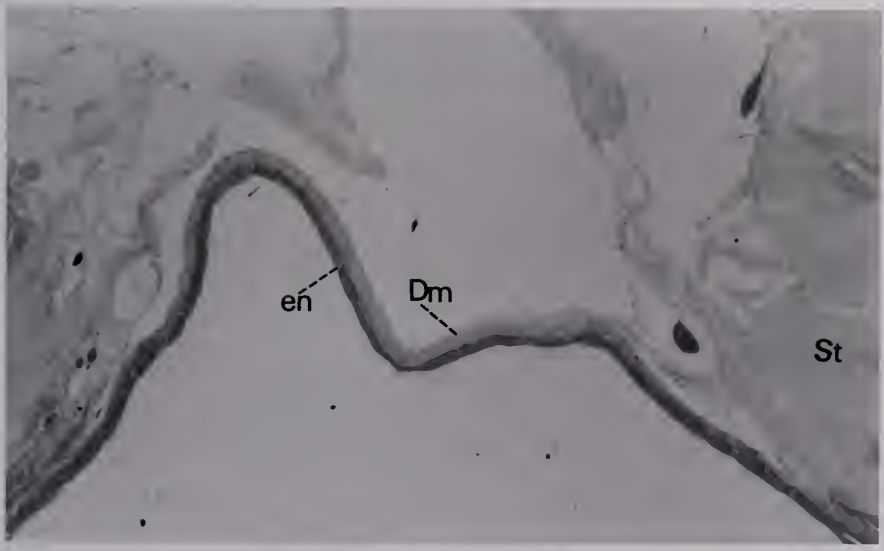

en
Dm
St

Figure 39. Electron photomicrograph of irradiated corneal epithelium covering nine week wound. Intracellular spaces (ics) are extensively enlarged but still bridged by desmosomes (ds). Healing inhibition is still apparent as indexed by abnormal epithelial organelles, lack of endoplasmic reticulum and a poor density of cytoplasm. (x 10,000)

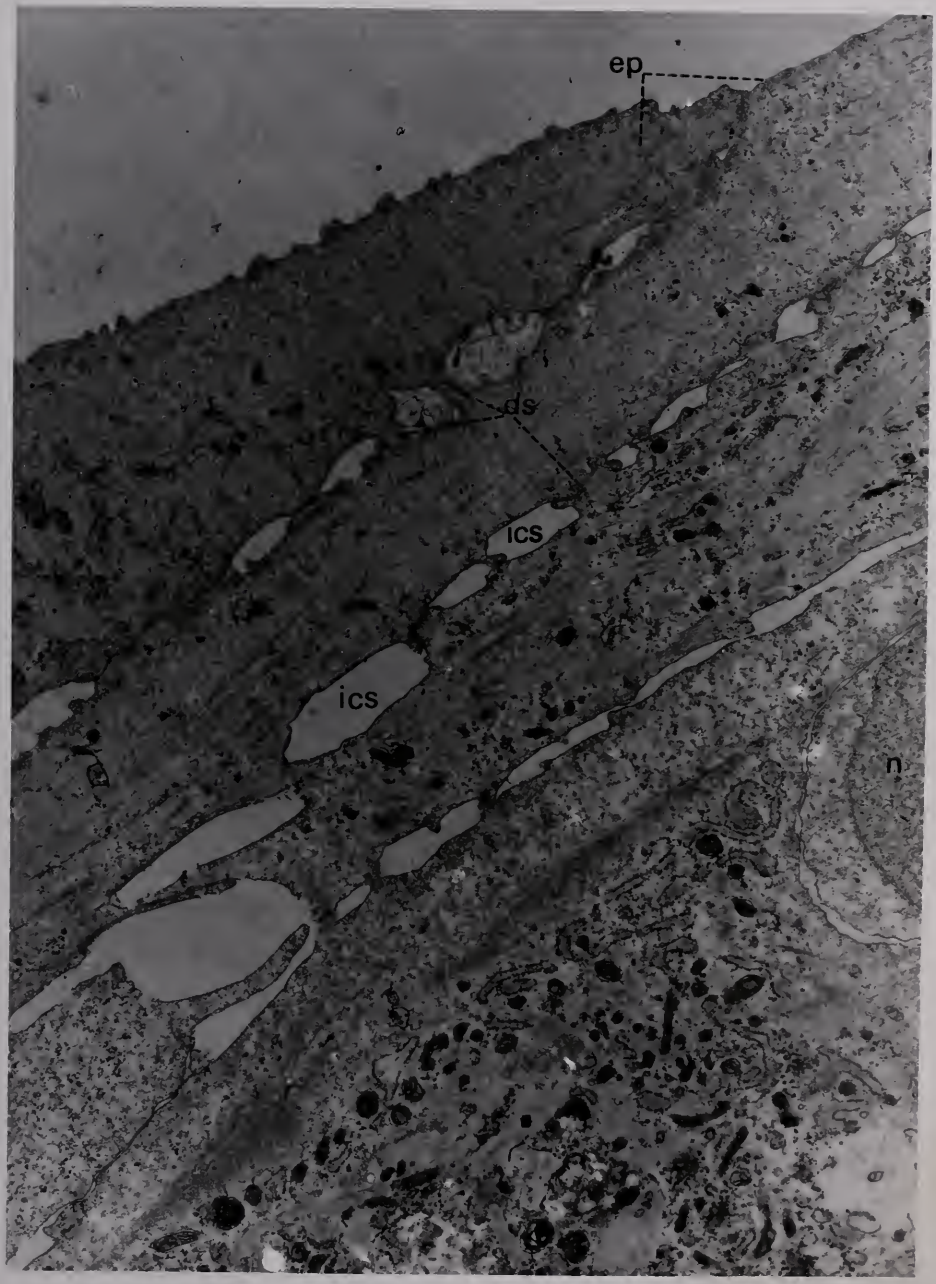

ep
ds
ics
ics
n

Figure 40. Electron photomicrograph of anterior stroma in irradiated cornea after nine weeks of healing. In this cornea, irradiated 10 months before wounding, cells are observed that are more like true fibroblast than the fibroblast-like cells noted in severely inhibited healing. (x 10,000; G = Golgi apparatus, nl = nucleolus, coll = collagen, m = mitochondria, rer = rough endoplasmic reticula)

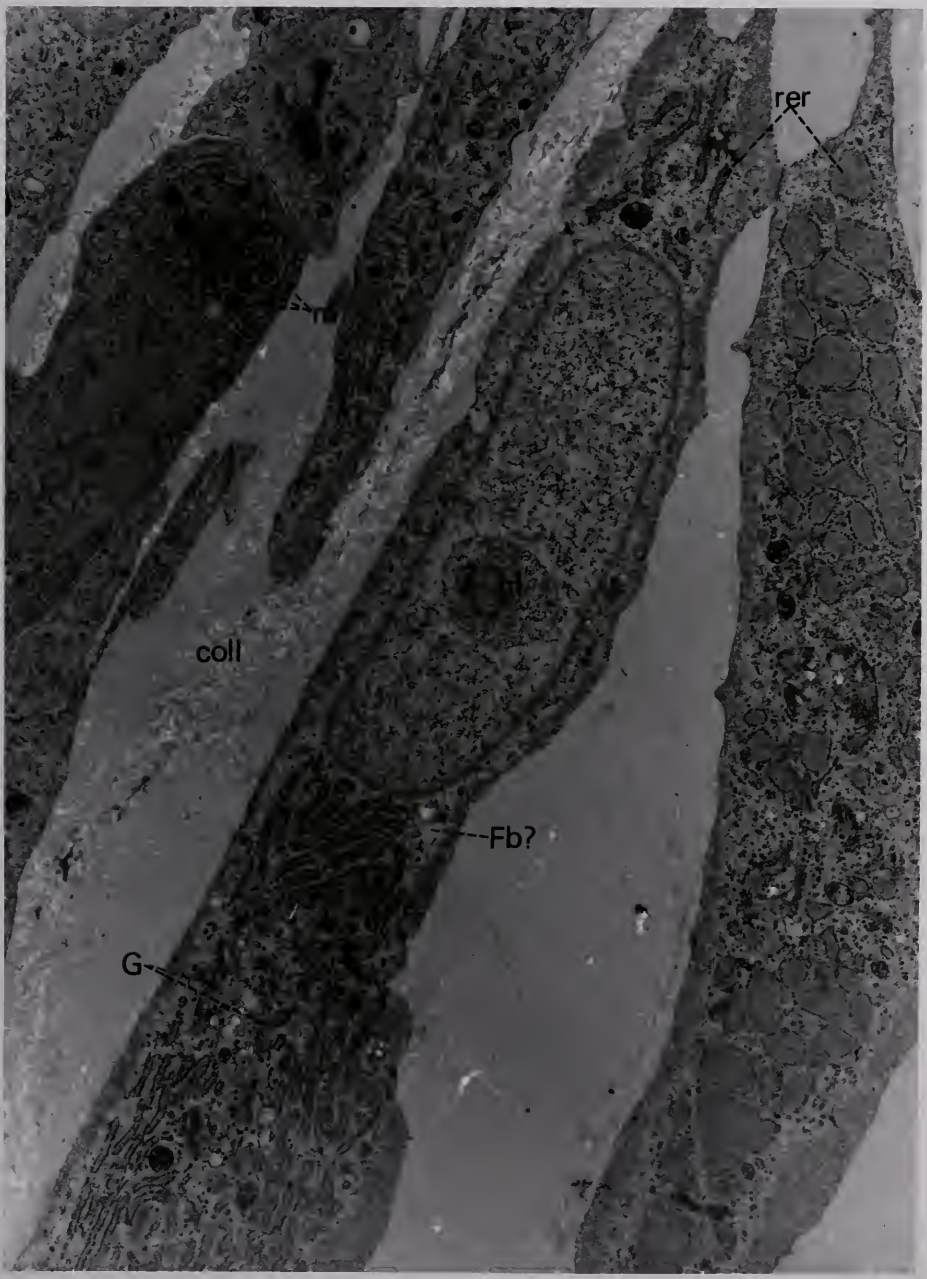

rer
coll
Fb?
G

Figure 41. Electron photomicrograph of stromal cells in nine-week wound in cornea irradiated ten months before wounding. The appearance of more normal fibroblast (Fb) is noted concomitantly with the appearance of new collagen (coll). (x 7,500)

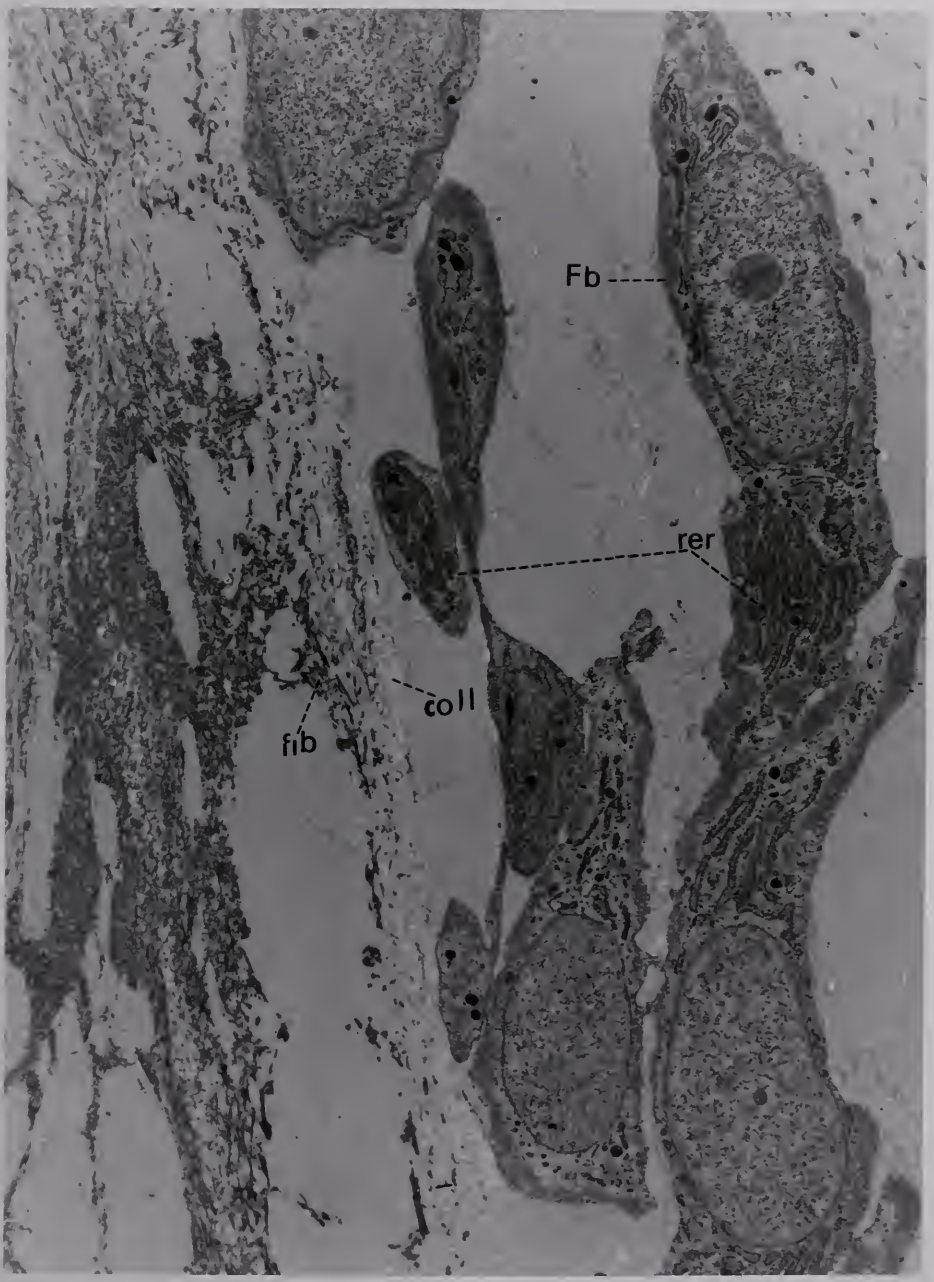

Fb
rer
coll
fib

Figure 42. Electron photomicrograph of collagen and fibrin in nine-week wound of irradiated cornea. Collagen fibers (cf) are much more widespread after ten months recovery from the radiation damage. (x 42,500, PTA stain)

cf
fib

Figure 43. Electron photomicrograph showing detailed fine structure of endothelium at nine weeks of healing in irradiated wound. Detailed fine structure of endothelium is still not normal however, an amorphous substance (probably Descemet's membrane) is being secreted along stromal side of endothelial cell membrane. Newly synthesized collagen is also noted in the stroma. (x 50,000, PTA stain)

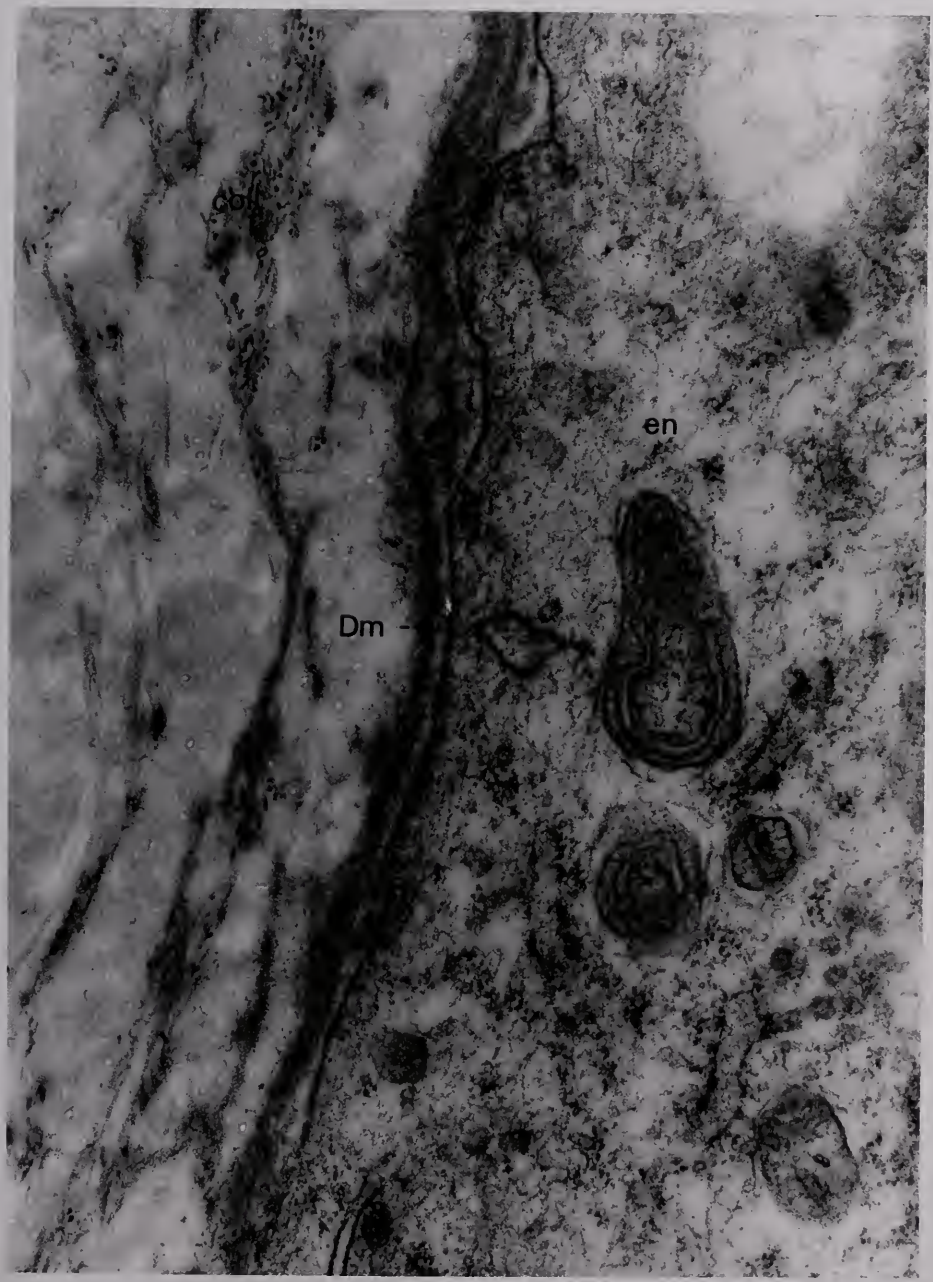

Cbl
en
Dm

Figure 44. Electron photomicrograph of endothelium and regenerating Descemet's membrane in nine week wound of control cornea. Descemet's membrane (Dm) is being replaced and newly synthesizing stromal collagen (coll) is apparently being organized. Note keratocyte presence in posterior stroma. (x 12,000)

en
n
Dm
coll
Kr

Figure 45. Electron photomicrograph of endothelium covering nine-week wound of irradiated cornea. Cornea received radiation treatments ten months before wounding. Inhibition of healing is marked by abnormally large intracellular spaces (compare with Figure 44), the absence of Descemet's membrane regeneration, and almost no stromal collagen formation. (x7,500, ics = intercellular space, St = stroma, n = nucleus)

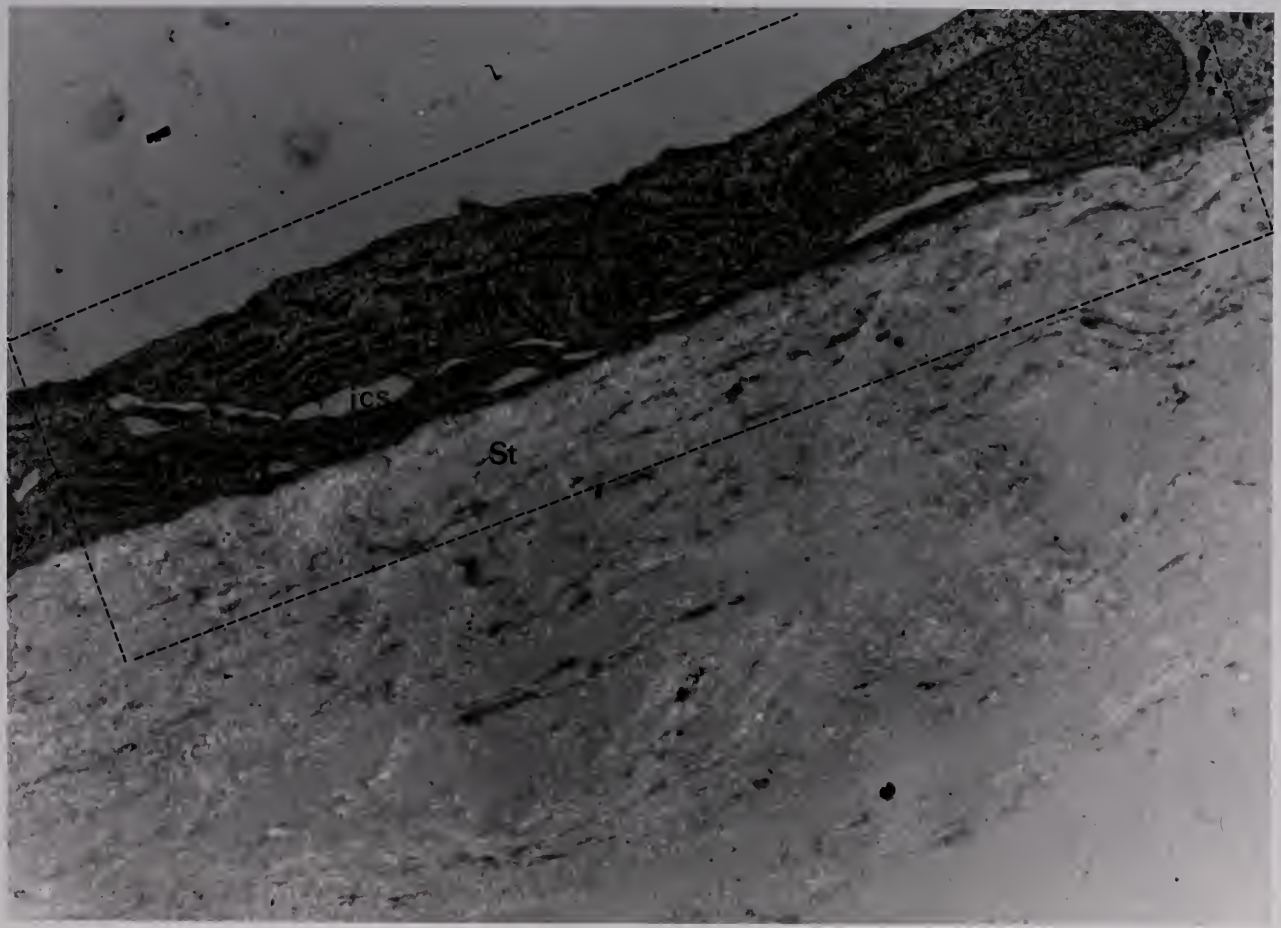
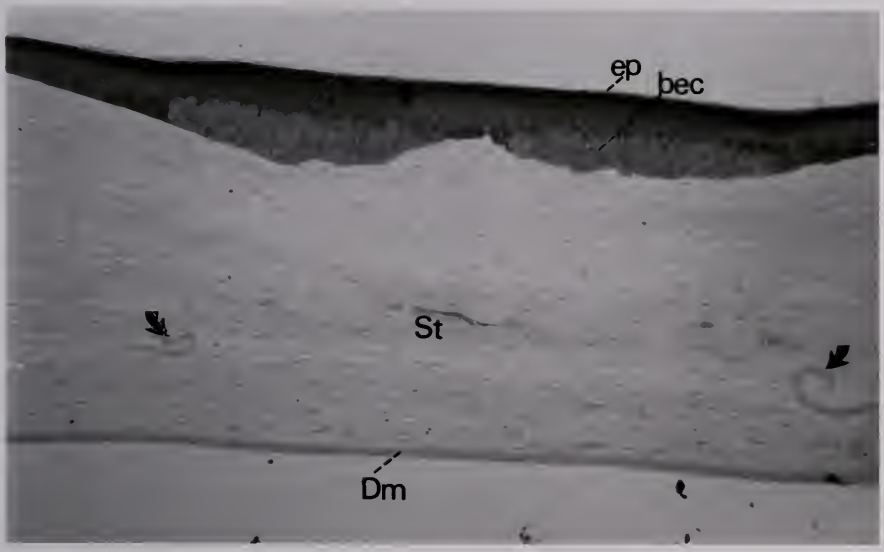

ics
St

Figure 46. Photomicrograph of control wound after nine months of healing. Stromal regeneration in the posterior wound is complete. Endothelium and Descemet's membrane appear normal. Except for the severed ends of Descemet's membrane (arrows) it would seem that the original incision had not been penetrating, actually normal healing has reconstructed the lamellar architecture in the posterior wound. The anterior wound, although regenerated, is still somewhat disorganized. Epithelium (ep) appears normal except for thickened area made up of basal epithelial cells (bec). (x100)

Figure 47. Photomicrograph of nine month wound in cornea irradiated three months before surgery. Disorganized arrangement of stromal wound extends all the way to the posterior of the wound. Note the paucity of cells involved in the wound area. (x100)


ep
bec
St
Dm

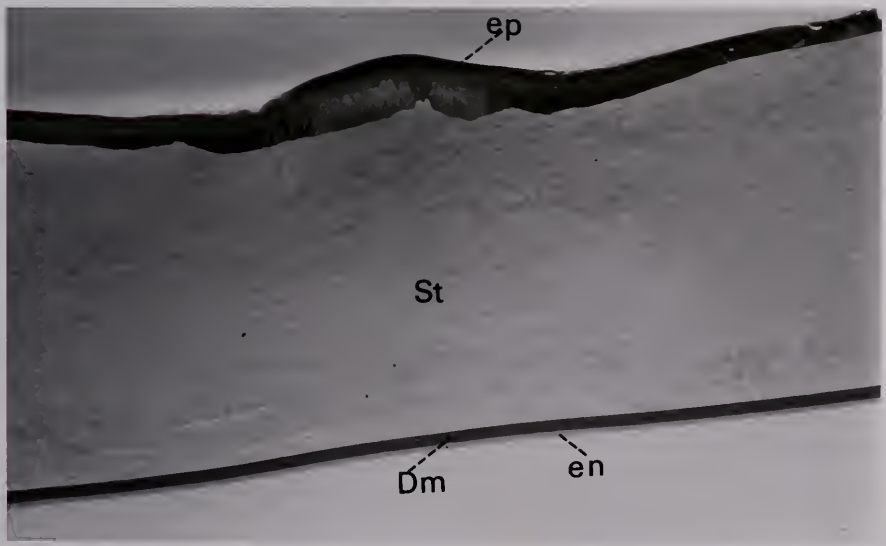

ep
St
Dm
en

Figure 48. Electron photomicrograph of stroma after nine months of healing in pre-irradiated cornea (PTA stain). Collagen fibers are reorganized into lamellae (Lm) typical of normal corneal architecture. Note keratocytes interposed between lamellae. (x 5,000)

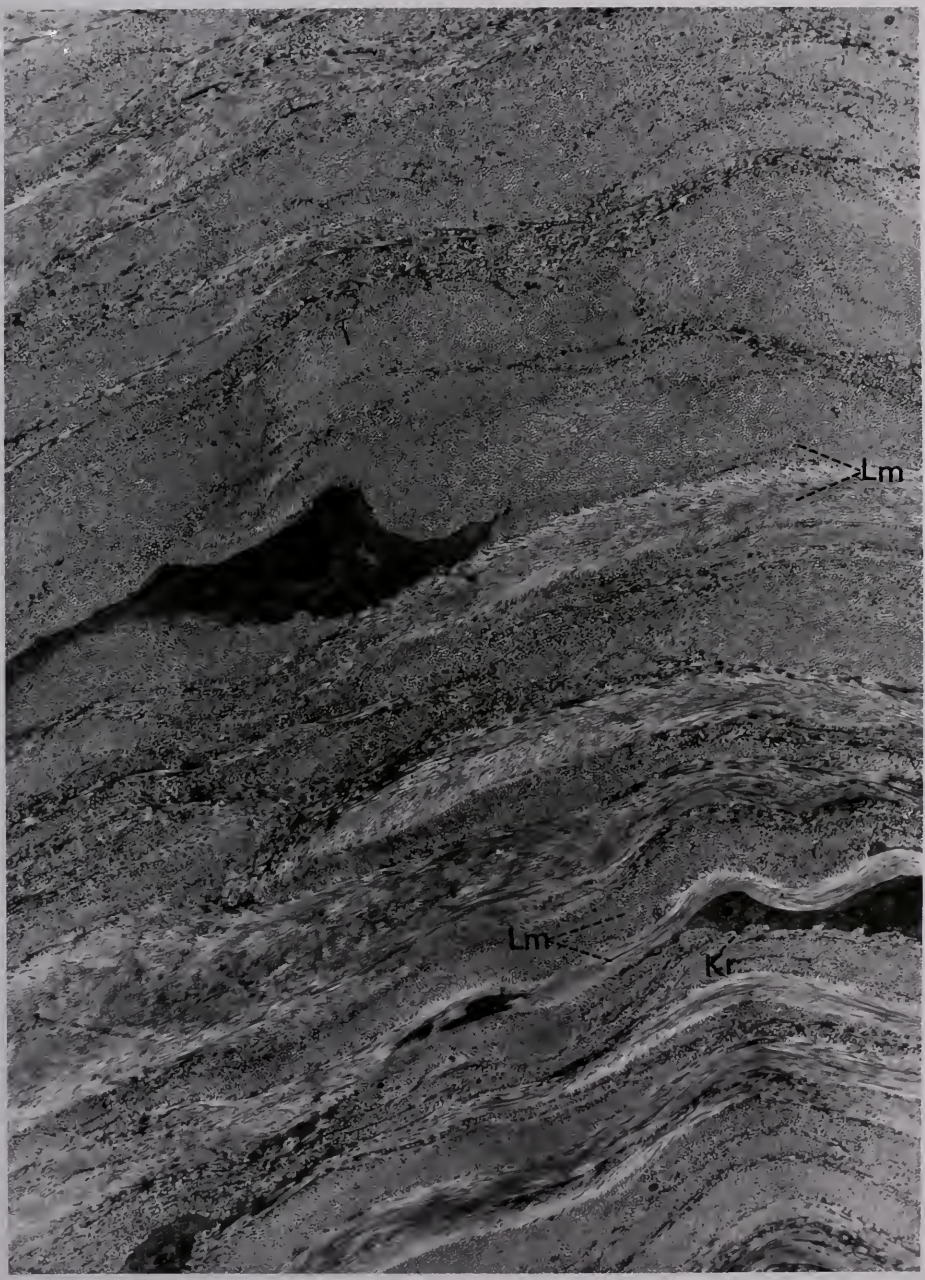
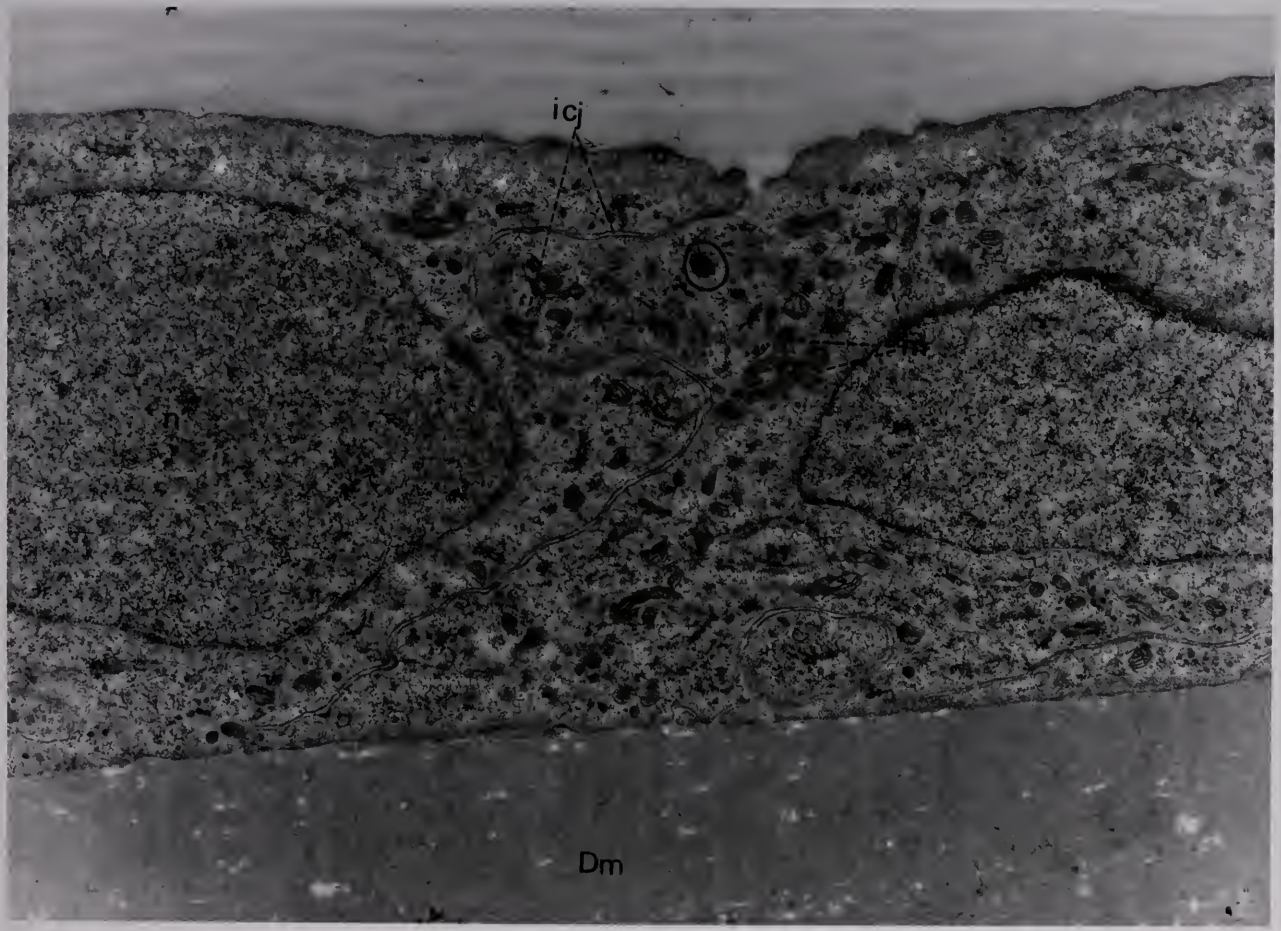

Lm
Lm
Kr

Figure 49. Electron photomicrograph of endothelium and Descemet's membrane covering nine month wound in pre-irradiated cornea (PTA stain). Endothelium is normal and Descemet's membrane has been regenerated. (x 5,000)

en
Om
St

Figure 50. Electron photomicrograph of endothelial ultrastructure after nine months of healing in irradiated cornea. Healed endothelium appears normal and intercellular junctions interdigitated. (x 15,000)


icj
n
Dm

SECTION 5

ENZYME HISTOCHEMISTRY

Enzyme Activity in Corneal Healing

The profound metabolic changes that occur during the initial phases of corneal healing provide an excellent opportunity to characterize some of the biochemical effects of beta radiation on this repair system.

The interrelationships of the epithelium and the other layers of the cornea involve such a variety of systems that partial inhibition of the functional biochemical integrity of any of these cell layers could have far reaching consequences in the complicated process of corneal healing.

In addition to the increased activity of succinic dehydrogenase, 5-nucleotidase, the formalin resistant oxidase that appears shortly after injury of the cornea, keratocytes demonstrate the ability to take up the vital dyė, Neutral Red, during the early stages of healing (93). This ability is dependent on the presence and functional integrity of the corneal epithelium and is inhibited by a variety of purine and pyrimidine analogues (94). Within one hour after wounding proteolytic enzyme activity develops in the injured cornea (95). The proteolytic enzymes are thought to disrupt lysozymes by

digestion of lipoprotein membranes, releasing hydrolytic enzymes that may alter the normal enzyme patterns of the cells. A functionally competent epithelium is required for stromal incorporation of sulfate into mucopolysaccharides (10). Since sulfate-transferring enzymes are reported to transfer sulfate to 3-phosphoadenosine-5'-phosphate and then to mucopolysaccharides (96), the biochemical interrelationships of the epithelium and stroma may play a significant role in the synthesis of new mucopolysaccharide ground substances.

In the preceeding sections, it was mentioned that beta radiation apparently had only a limited effect on epithelial function and multiplication. In spite of the abnormal morphology and layering of the epithelial cells, they seemed to retain the ability to expand, slide over the wound, and eventually repopulate the epithelial portion of the defect. Although the effects of beta radiation were not severe enough to completely inhibit epithelial cell function, the changes noted suggest that the beta radiation could compromise the normal interrelationships between epithelial cells and other cellular activities involved in corneal healing.

Radiation induced changes in the enzymic equilibrium of the epithelium of the rabbit lens have been shown to occur within 18 hours after 800 r. of X-irradiation (97). Other changes, in glycolysis (98) and albuminoid protein breakdown (99) in lens tissues, have been followed for several weeks after radiation exposure. Beta radiation has a greater biological effectiveness than X-radiation, thus, one would expect that doses of several thousand rads of beta

radiation administered to the cornea would have some effect on local enzyme activity.

Considering the complexity of the enzyme patterns that are particularly associated with corneal healing, it would seem pertinent to evaluate the biochemical capability of the different cell layers to elucidate any radiation induced changes that might influence the functional capability of other cell layers.

Methods

Substrate specific histochemical techniques were used to localize the active sites of particular enzymatic activities. Three eyes from each group were bisected perpendicular to the wound. Following autoradiographic labeling, one-half of each eye was frozen for enzyme studies while the other half was fixed and embedded for autoradiographic studies. Immediately after enucleation, the eyes were immersed in a mixture of isopentane and liquid nitrogen at a temperature of -120° C. for several minutes, and then stored a -70° C. Frozen sections were cut on an International cryostat and kept at -70° C. until incubation. Two sections were placed on each slide: one each of the irradiated eye and of the contralateral control eye. Multiple slides were stained by separate methods for succinic dehydrogenase, lactic dehydrogenase, $NADH_2$ diaphorase, glyceraldehyde-3- phosphate dehydrogenase, and 5-nucleotidase activities. Microscopic examination of the sections thus stained revealed the sites of diformazan precipitate indicating the location of the enzyme activity.

Succinic dehydrogenase

Nitro-BT (a tetrazolium compound) will act as an electron acceptor to replace the flavin based acceptors normally found as coenzymes in succinic dehydrogenase enzymatic reactions. The Nitro-BT accepts the electron and forms a diformazan, insoluble precipitate at the site of enzymatic activity, whenever the appropriate substrate is included in the incubate (100). The system has been shown to be substrate specific at pH's of 7.6 and below (101).

Modification of the standard incubation solutions included the addition of phenazine methsulfonate which acts as an intermediary between the enzyme substrate and the Nitro-BT. The addition of this compound reduced the incubation times from six hours to thirty minutes at 37° C.

The stock solution of nitroblue-tetrazolium is prepared in 0.25 M phosphate buffer as follows: (1) To approximately 100 ml. of K_2HPO_4 (34.8 Gm./L.) slowly add a sufficient quantity of KH_2PO_4 solution (27.2 Gm./L.) to obtain a pH of 7.3. (2) In the phosphate buffer dissolve sufficient Nitro-BT to make a solution containing 0.3 mg./ml. of Nitro-BT. Store this stock solution in a refrigerator at 4° C.

The incubation solution for succinic dehydrogenase was prepared by mixing 15 ml. of Nitro-BT stock solution with 15 ml. of 0.2 M sodium succinate, then adding a freshly prepared solution of phenazine methsulfonate to give a final concentration in the incubate of 0.2 M. Control solutions of incubate were the same except that the sodium succinate substrate is left out. It should be noted that the phenazine

methsulfonate is extremely light sensitive and subject to degradation upon even short exposures of several minutes to normal light or excess heat. Therefore, all phenazine methsulfonate solutions were made up fresh and the incubation had to be carried out in the dark.

The frozen sections were allowed to thaw immediately prior to incubation. The sections were incubated at 37°C. for one hour in the dark. Control sections were incubated in the same incubation solutions minus the substrate to check for substrate specificity. If degradation of the phenazine methsulfonate causes formation of the diformazan precipitate from the incubation solutions, the color of the incubate changes from yellow to green. This color change indicates an unsatisfactory incubation solution.

Following incubation, the slides containing the tissue sections were rinsed in distilled water and fixed in 10 percent buffered formalin. They were able to retain the stain for seven to ten months following this. Once the sections were fixed for 30 minutes or more, they were rinsed in distilled water and coverslipped using a flycerin base mounting media.

A counterstain was developed for better contrast of the dark blue diformazan precipitate. Nuclear Fast Red gave good contrast and permitted observation of the orientation of the nuclei for better illustration of the location of the diformazan indicator.

Lactic acid dehydrogenase

The same system of Nitro-BT incubation was used with sodium lactate to determine the LDH activity in converting lactate to pyruvate. NAD is the normal coenzyme found in LDH conversion of

lactate; hence, it was used as the coenzyme in place of phenazine methsulfonate.

The incubate was made up of 10 ml. Nitro-BT solution, 5 ml. of 0.005 M NAD, 0.4 ml. of a 60 percent aqueous solution of sodium lactate, and 30 ml. triple distilled water. The corneal sections on microscope slides were incubated for one hour at 60° C. and compared with similar sections incubated in the same solution minus the sodium lactate substrate.

$NADH_2$-diaphorase

The Nitro-BT system was also used to locate the diaphorase enzymes that utilize the reduced form of NAD ($NADH_2$) as a substrate.

Frozen sections were thawed, and incubated in a solution of 15 ml. of Nitro-BT stock solution, 5 ml. of 0.005 M $NADH_2$, and 30 ml. triple distilled water for 30 minutes at 37° C. in the dark. Control incubations in the Nitro-BT stock solution diluted with tripled distilled water were used to check substrate specificity.

Glyceraldehyde-3-phosphate dehydrogenase

Conversion of glyceraldehyde-3-phosphate (GAP) to 1, 3-diphosphoglyceraldehyde represents a simple dehydrogenase reaction that is the sole oxidative step in the Embden-Meyerhof glycolytic pathway.

The Nitro-BT coupled reaction was used to determine the activity of glyceraldehyde-3-phosphate dehydrogenase in the corneas.

Initial attempts to localize diformazan precipitation resulting from GAP dehydrogenase activity used 15 ml. of Nitro-BT stock solution, 10 ml. of 0.1 M GAP, and 3 ml. of 0.005 M NAD at 37° C. as the incubate.

The results were not substrate specific even though several different incubation times were tried between 30 minutes and 2 hours.

Another attempt utilized the same mixture; however, 0.5 cc of 0.2 M phenazine methsulfonate was added and temperatures were varied from 37° C. to 60° C. Incubation times of 45 minutes or longer at 60° C. caused spontaneous precipitation of diformazan out of the solutions. Incubation for one hour at 37° C. followed by 30 minutes at 60° C. was promising, but substrate specificity could not be verified in all of the several trials.

Other modifications were tried — (1) Lipid can cause precipitation of diformazan out of Nitro-BT solutions containing pyridine nucleotides(102); hence, a 20 minute soak in acetone at 2° C. to remove lipids was added to the procedure before incubation of the sections. (2) GAP dehydrogenase enzymes contain some labile sulfhydryl groups that are easily deactivated. Cysteine will stabilize these sulfhydryl groups and preserve the activity of the enzyme (103). Sections were activated in 0.2 M cysteine following acetone soak, prior to incubation. (3) At low enzyme concentrations in the presence of GAP, no enzyme activity is demonstrable unless pyrophosphate or arsenate is present(103). The cysteine was dissolved in 0.1 M sodium pyrophosphate adjusted to pH 8.4 with HCl.

In spite of modifications, to allow for lipid removal, sulfhydryl stabilization and pyrophosphate as an inorganic source of phosphorous, the Nitro Blue Tetrazolium coupled reaction was not substrate specific for glyceraldehyde-3-phosphate dehydrogenase activity.

5-Nucleotidase

Adenosine-5-phosphate is the substrate for 5-Nucleotidase activity in tissues. A reaction product was formed by incubation of frozen sections in a mixture containing 30 ml. of adenosine-5-phosphate solution (78.7 ml./100 ml.); 30 ml. of 0.04 M Tris buffer (pH 7.2); 7.5 ml. of 0.1 M $MnCl_2$ solution; 4.5 ml. of 2 percent $Pb(NO_3)_2$; and 3 ml. of triple distilled water. This mixture was made up fresh for each experiment and filtered through Whatman No. 42 filter paper before use. Control sections were incubated in the same mixture minus adenosine-5-phosphate.

The incubation was carried out at 37° C. for five hours, followed by fixation in 37 percent formalin for 30 minutes, rinsed in distilled water, immersed for two minutes in 2 percent (v/v) NH_4S solution, rinsed again in distilled water and then counter-stained in 0.2 percent unextracted methyl green in 0.2 M acetate buffer (pH 4.0) for two minutes.

The sections were next destained in two rinses of absolute ethyl alcohol for ten seconds each, cleared in toluene for ten minutes and cedarwood oil for ten minutes and mounted in Permount.

Valid comparisons of the difference in enzyme activities between non-irradiated control wounds and irradiated wounds, in the contra-lateral eye, were made possible by the simultaneous staining of sections from each eye placed on the same slide and processed together.

Results

Briefly, the enzyme histochemical findings indicated a reproduceable, consistant decrease in succinic dehydrogenase activity associated with corneal repair in those eyes that had received beta radiation either before or after surgery. No discernable differences were evident in lactic acid dehydrogenase (LDH), 5-Nucleotidase, nor $NADH_2$-diaphorase activities in the irradiated corneas when compared to non-irradiated corneas. Even after considerable modifications of the histochemical techniques, glyceraldehyde-3-phosphate dehydrogenase (GAP-DH) activities demonstrated in the tissues could not be consistently verified as being substrate specific. Therefore, GAP-DH activities were not compared for irradiated and non-irradiated corneal wounds. The detailed findings are described below.

Succinic dehydrogenase activity

Corneal tissue samples were taken for succinic dehydrogenase (SDH) activity determinations at 18, 24, 42, and 96 hours and 5, 10, and 20 days following surgery in the groups of animals that received beta radiation immediately after surgery. In the groups that received beta radiation prior to surgery (two and one-half or ten months before) the eyes were enucleated at 24, 48, 72, and 96 hours and 10 and 20 days after wounding.

Non-irradiated corneas. — Control sections incubated in the substrate solution lacking succinate were always negative. In the non-irradiated corneas the enzyme activity was limited to a few infiltrating polymorphonuclear leukocytes up to 24 hours after surgery.

TABLE 3

EMPIRICAL EVALUATION OF SDH ACTIVITY

Treatment	Healing Time After Wounding							
	Hours					Days		
	24	42	48	72	96	5	10	20
Non-irradiated	1	2	2	3	4	4	4	2
	1	1	2	3	3	4	3	1
	1	2	1	3	4	4	4	2
	0	1	2	2	3	4	3	1
	0	0	1	2	3	3	3	2
	1	-	2	3	4	4	3	-
Mean Rating	0.7	1.2	1.7	2.7	3.5	3.8	3.2	1.6
Irradiated								
At surgery	0	0	-	-	1	1	1	0
	0	1	-	-	2	2	1	0
Two and one-half months before surgery	0	0	1	1	1	2	2	1
	0	0	0	2	2	2	1	0
Ten months before surgery	-	-	1	-	1	2	1	1
	-	-	1	-	3	3	2	1
Mean Rating		0.3	0.8	1.5	1.6	2.0	1.3	0.8

SDH activity within 42 to 48 hours after injury was demonstrable in some of the keratocytes near the wound, in some of the fibroblasts at the wound edge, and occasionally some faint activity was noted in the epithelium at the wound site.

Epithelial activity had increased by four days of healing, but all activity was still confined to the wound area. SDH activity in the stroma was also limited to a narrow band about the wound, but at this time it was a wider band of active cells than noted in earlier samples. The most activity was found in the nuclei of stromal cells involved in the wound site and invading mononuclear cells nearest the wound. The fibroblasts at the wound perimeter were also very active at this time.

The heaviest concentrations of enzyme activity were found at day five in the same cells and in the same general locations as found a day earlier. Epithelial activity was the greatest at this time of all periods examined. Fibroblasts involved in the wound were very rich in activity, as were keratocytes in the immediate vicinity of the wound.

As healing time progressed the area of highest activity seemed to narrow to a small margin around the wound edge. By day ten the epithelium was active only in the immediate vicinity of the wound and the stromal area containing very active cells was confined to a narrow band of about 250 to 300 microns on either side of the wound. In this active region the fibroblasts were still very active; however, the activity seemed less than the level of activity observed in the five-

day-old wounds. A similar decrease was noted in the level of activity of the keratocytes contained in the wound vicinity. After 20 days of healing the succinic dehydrogenase activity in the non-irradiated control wounds was considerably less in all cells than those levels of activity found at three or four days. The distribution was again confined to a narrow band along the wound perimeter, but by this time, epithelial activity was almost negligible.

Irradiated corneas. — In eyes that were irradiated either immediately after surgery or two and one-half months prior to surgery, there was a substantial decrease in the succinic dehydrogenase activity of all cells associated with the wound. Subjective evaluations of this decrease, using an empirical grading scale of zero to four-plus, indicated that those corneas that received beta radiation ten months before wounding demonstrated more SDH activity than those irradiated two and one-half months before, or immediately after wounding. The levels in these corneas that were irradiated ten months before surgery were not as high as found in the contralateral non-irradiated corneas, however.

In addition to the overall decrease in SDH activity noted in the irradiated corneas, it was apparent that the SDH activity that was present developed later than in the non-irradiated corneas.

No activity was found in any of the 24-hour-old wounds. In a few sections at 42 to 48 hours of healing, some activity was noted in a few stromal cells, predominantly keratocytes, but this SDH activity was minimal and not well defined. By 72 to 96 hours, most

corneas examined had some SDH activity confined to the immediate wound area. This activity was found in keratocytes, fibroblasts, and some invading mononuclear cells, but only occasional sections showed epithelial SDH activity.

Peak activity was noted in the irradiated corneas at day five after wounding. Compared to the non-irradiated corneas at the same period of healing, the irradiated corneas demonstrated substantially less activity overall. The SDH staining decreased in the corneas by ten days of healing and was barely discernable in the corneas enucleated 20 days after wounding.

There was a trend in the treated corneas suggesting that the beta radiation not only caused a reduction in the overall SDH enzymatic capability of the cells, but also produced a delay in the onset of demonstrable activity associated with healing of the wounds.

Lactic dehydrogenase activity

Lactic dehydrogenase (LDH) activity was determined in wounded corneas enucleated at 24, 48, 72, and 96 hours and 5 and 10 days following surgery.

Non-irradiated corneas. —— The LDH reaction converting lactate to pyruvate was demonstrable in the epithelium at all times examined during healing. The keratocytes showed some minimal activity by 48 hours; however, active cells were not restricted to the immediate wound area. LDH activity was increased in 72 and 96 hours of healing and many keratocytes in the more peripheral stroma were found to be active. Between four and five days of healing epithelial activity also seemed to reach maximum levels.

Irradiated corneas. — No consistent differences in LDH activities were detectable between the irradiated and the non-irradiated corneal wounds. LDH activity levels were approximately the same at any given time in the sequence studied.

It should be noted that frequently, when comparing sections from irradiated and non-irradiated corneas from the same animal, it seemed that the irradiated corneas demonstrated more LDH activity than the non-irradiated controls. These observations were not consistent among all animals evaluated. In the absence of quantitative measurements, these slight differences, although notable, were not considered to be valid proof of a demonstrable difference in LDH activity between the irradiated and non-irradiated corneal wounds.

$NADH_2$ diaphorase activity

Corneal wounds were evaluated for $NADH_2$ diaphorase at the same periods of healing as the above mentioned LDH evaluations. It was observed that considerable diaphorase activity was present in epithelial cells and in stromal keratocytes during all periods examined. Although activity was slightly more pronounced in epithelial cells than in keratocytes, overall levels did not vary as a function of healing time.

There was no demonstrable difference in $NADH_2$ diaphorase activities found in irradiated corneas when compared to non-irradiated control corneas.

Glyceraldehyde-3-phosphate dehydrogenase activity

Unfortuantely all attempts to identify sites of GAP-DH activity in wounded corneas have not successfully established a technique that is reproduceably substrate specific. Therefore, no results are

reported herein as to location and relative levels of GAP dehydrogenase activity in corneal wounds.

5-nucleotidase activity

Generally, non-irradiated and irradiated corneal wounds demonstrated 5-nucleotidase activities similar to those reported previously (29). Few differences were attributed to the effects of beta radiation on the induced 5-nucleotidase activity in the injured cornea.

5-nucleotidase activity was discernable in many stromal cells by 18 hours. These active cells were not located in any one area relative to the wound. Some slightly active keratocytes were observed as far away from the wound edge as the limbus. By 48 hours of healing the most intensely active cells were found in the wound area. Cells peripheral to the wound edge were less and less active as one moved farther away.

Basal epithelial cell activity was noted at 72 hours of healing. At 96 hours, the first 5-nucleotidase activity in some of the endothelial cells was observed. Maximal activity in epithelium and endothelium was not apparent until five or ten days after wounding. The endothelium had not recovered the wound posteriorly in the irradiated corneas so none of the active endothelial cells were found directly involved in the wound site.

Enzyme activity in the five and ten day wounds was restricted to a narrow band approximately 300 to 400 microns wide, on either side of the wound edge. Fibroblasts were very active at this time in the immediate wound area.

The only difference between irradiated and non-irradiated corneal wounds was noted at five and ten days after injury. At these times, fewer numbers of active fibroblasts and greater numbers of active polymorphonuclear leukocytes were observed in the irradiated wounds than were observed in the non-irradiated control wounds. The total activity in the wound area was about the same, however, in spite of the differences in numbers of different types of cells that were found active. By day 20 this difference was no longer noticeable and overall 5-nucleotidase activity was considerably less than in the ten day samples. Apparently, the peak 5-nucleotidase activity had begun to diminish by 20 days of healing.

Comment

Succinic dehydrogenase and 5-nucleotidase activities are not demonstrable in normal corneas (29). A distinctive pattern of these enzymatic activities is present in the wounded cornea during the first several weeks of healing. It seems reasonable to consider that these enzymes are somehow essential to the overall enzymic interactions found in the corneal healing phenomenon. It has been proposed that the substrate for 5-nucleotidase, adenosine-5'-phosphate, is a storage form of "active" adenine, a precursor of RNA synthesis (104). The function of 5-nucleotidase in the normal cell is still unknown, however, since increased RNA synthesis has been demonstrated in normal corneal healing (32) at about the same time as increased 5-nucleotidase activity, it is interesting to speculate that the two might be related.

Succinic dehydrogenase is a unique enzyme found in the mitochondrial fraction of the cell and closely associated with the respiratory enzyme chain. SDH catalyses the only dehydrogenation reaction in the citric acid cycle that involves a direct transfer of hydrogen from the substrate to a flavoprotein (FAD) without involving NAD as an intermediate (105).

In the ultrastructural studies mentioned previously in Section 4, it was shown that the beta radiation produced changes in the mitochondrial and other cellular organelles of the cells involved in the healing process in the cornea. It would be important to know if mitochondrial function is also changed.

If SDH activity is inhibited, the conversion of succinate to fumarate is inhibited, resulting in a block of the cyclic role of acetate in the citric acid cycle. Succinate will accumulate unless it is utilized in some other way. In animals it can only be utilized in one other major pathway, as "active" (CoA) succinate, by condensation with glycine to form alpha-amino-beta-ketoadipic acid which is converted to precursors for porphyrin synthesis.

Interference with SDH activity in a cell, therefore, can decrease the metabolic energy produced by the citric acid cycle (TCA cycle) linked with oxidative phosphorylation in the mitochondria. This would reduce the efficiency of aerobic catabolism of glucose, since the citric acid cycle provides 30 of the 38 high energy phosphate bonds (ATP's) generated per mole of glucose oxidized to CO_2 and water. Although cytochrome oxidase activity has not been found in the healing

cornea (29), another oxidase has been demonstrated (30). It is reasonable to assume that under the increased metabolic demands of corneal healing, the maximum efficiency of glucose utilization would be required. Therefore, a normally functioning citric acid cycle may be very important to the repair capability of the cells involved in corneal healing. Since beta radiation decreases the high levels of succinic dehydrogenase activity found in the healing cornea, the radiation might well be responsible for a reduction in the overall metabolic energy available for wound repair in the cornea.

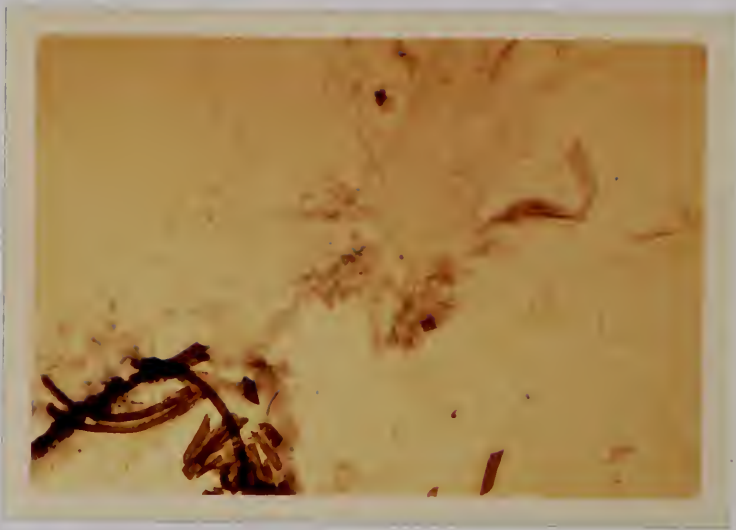
Knowing that the citric acid cycle might well be inhibited by the beta radiation effects on SDH activity and that beta radiation resulted in mitochondrial abnormalities, it seemed pertinent to investigate the aerobic glycolytic pathway for normal functional capability outside the mitochondria. Glyceraldehyde-3-phosphate dehydrogenase (GAP-DH) was selected as an index of normal aerobic glycolysis via the Embden-Meyerhof scheme. This enzyme catalyses the only oxidative reaction in the entire sequence of glucose conversion to pyruvate that is linked with respiratory chain oxidation via NAD. Unfortunately, the substrate specific activity of GAP-DH was not demonstrated by the techniques employed in these experiments. Assay methods involving isolation, purification, and quantitative analysis of GAP-DH were not within the scope of this research at this time; therefore, the extramitochondrial portion of glucose utilization was not evaluated.

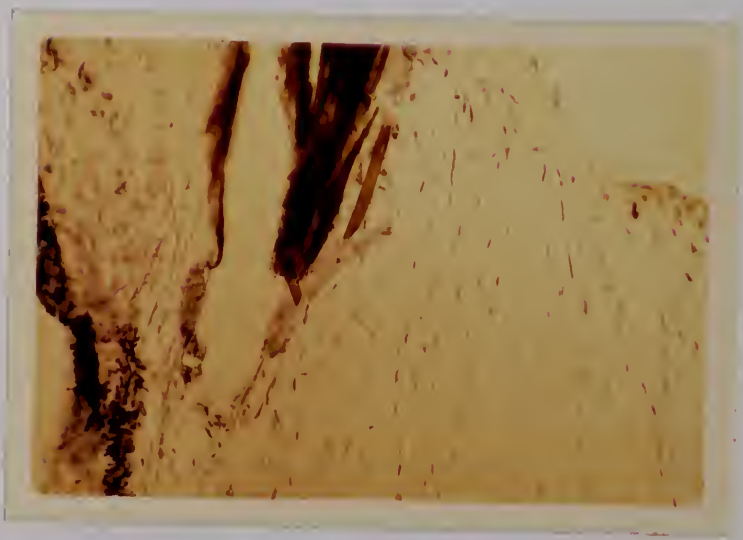
Lactic dehydrogenase (LDH) plays a key role in anerobic glycolysis by conversion pyruvate to lactate with the concomitant oxidation of $NADH_2$ to NAD, which is utilized by GAP-DH to keep glycolysis functioning in the absence of NAD supplied by the respiratory chain enzymes. Since the Nitro-BT histochemical techniques are applicable only to dehydrogenase reactions involving the liberation of a hydrogen, and the LDH conversion of pyruvate to lactate involves the transfer of a hydrogen from $NADH_2$ to pyruvate, this system could not be used to determine LDH activity in the formation of lactate. Instead, LDH activity for the reverse reaction (conversion of lactate to pyruvate with NAD as the coenzyme) and the activity of normal $NADH_2$ diaphorase was determined in an effort to evaluate any radiation effect on the LDH reactions involving pyruvate and lactate.

Both LDH and $NADH_2$ diaphorase activities in the irradiated corneal wounds appeared to be comparable to the activities demonstrated in the non-irradiated corneas. Although there was some suggestion of increased LDH activity in the irradiated corneal wounds this increase remains to be verified by other, quantitative, methods of evaluating lactate and pyruvate utilization, *in vivo*, in irradiated and non-irradiated corneal wounds.

Figure 51. Photomicrograph of non-irradiated wound showing Succinic Dehydrogenase activity (Nitro-BT stain). Suture material is shown in lower left-hand corner, no counterstain was used. Diformzan precipate is found only where the enzyme is active. (x 100)

Figure 52. Photomicrograph of non-irradiated corneal wound demonstrating Lactic Dehydrogenase activity (Nitro-BT stain). Stromal keratocyte activity is obvious. Note suture in top center of picture. (x 250)





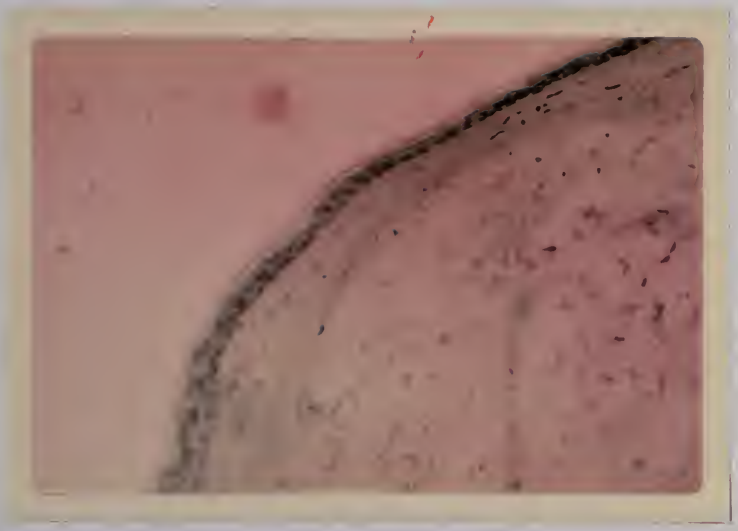


Figure 53. Photomicrograph illustrating histochemical staining of 5-nucleotidase in non-irradiated corneal wound. (x 250)

SECTION 6

NUCLEIC ACID AND MUCOPOLYSACCHARIDE SYNTHESIS

In the previous experiments on corneal wound healing it was determined that the beta radiation treatments (1) effected epithelial morphology and enzymatic activity, (2) inhibited the appearance of mature stromal fibroblasts and inhibited stromal collagen synthesis, thereby prohibiting the normal development of tensile strength of the corneal wounds, and (3) inhibited the migration of endothelial cells, thus preventing closure of the wound posteriorly and probably producing drastic secondary effects on stromal and endothelial metabolism.

The radiation induced ultrastructural changes noted in the corneal wound had some interesting implications. Particularly, in fibroblast-like cells, the substantial lack of rough endoplasmic reticulum (ER) was correlated with the absence of collagen synthesis, normally associated with fibroblastic appearance and proliferation. Further, the variation and lack of rough ER before and after delayed endothelial migration suggested that some problem with cytoplasmic protein synthesis might be present. Current concepts of intracellular fine structure and its functions indicate that the ribosomes of the rough ER are the principal sites of protein synthesis within a cell. This has been demonstrated in fibroblasts involved in wound repair (106). The Golgi apparatus of the cells has also been implicated in protein

synthesis and polysaccharide synthesis (87). If the beta radiation-induced changes in ultrastructure were related to inhibition of protein and/or mucopolysaccharide synthesis then the radiation effects should encompass changes in the RNA synthesis which is essential for the formation of protein.

The preceeding experiments also emphasized the radiation inhibition of fibroblastic proliferation during the first several months of corneal healing. A substantial portion of the fibroblasts present in corneal wounds are throught to originate from mitotic division of other fibroblasts (19). It has been demonstrated that irradiation shortly after wounding results in a 50 percent reduction in the numbers of involved cells synthesizing DNA some five days after wounding (20).

Considering the information gained in the preceeding experiments of beta radiation effects on morphology and biochemical synthesis of the cells contributing to the healing process in the cornea, it seemed pertinent to study the beta radiation effects on RNA, DNA, and mucopolysaccharide synthesis in corneal repair.

Biological Basis of Labeled Precursor Uptake

There is a clear distinction between the total DNA or RNA content of a cell and the rate of biosynthesis, which can be studied by incorporation of radioisotope labeled selective precursors.

In the mitotic cycle of a cell the interphase period between successive mitotic divisions is divided into three different periods: G_1, S, and G_2. The G_1 period follows mitosis. DNA synthesis during the G_1 period is negligible and the DNA content of the cell is constant.

During the S, or synthesis period, the DNA content is doubled. Following DNA synthesis occurring during the S period, there is a "resting" period called G_2 during which the cell's DNA content is twice that of the G_1 period between mitotic divisions. DNA synthesis during the G_1 period is inhibited by radiation at lower doses than are required to inhibit DNA synthesis once it has begun (107). Applications of these relative radiosensitivities have shown that incorporation of tritiated thymidine into the DNA of fibroblasts is dramatically inhibited only if the majority of the fibroblasts are in the G_1 phase (108).

Different radiosensitivities exist among DNA, nuclear RNA, and cytoplasmic RNA of the same cell. Nuclear RNA synthesis is inhibited at lower doses of ionizing radiation than cytoplasmic RNA synthesis (109). In vitro experiments have shown that nuclear RNA synthesis is also more radiosensitive than DNA synthesis (110).

Tritiated thymidine has been used to study the DNA synthesis in corneal stromal cells following wounding (111). Tritiated thymidine incorporation into DNA synthesis in damaged stromal cells has been used as a basis for labeling corneal stromal cells prior to transplantation (112). These labeled cells were found to persist in corneal homografts in undiminished numbers for as long as 12 months (14).

The development of large numerous nucleoli in corneal keratocytes undergoing transformation into fibroblasts (37) has suggested that increased protein synthesis and RNA synthesis occurs in these cells during corneal repair (29). Tritiated uridine incorporation into RNA synthesis has been used to characterize the nuclear RNA synthetic patterns in corneal cells during the first eight days of healing (32).

These same studies delineated patterns of the DNA, protein, and sulfated mucopolysaccharide synthesis during the same period of corneal healing. Sulfur-35 uptake has been used to determine the location and approximate magnitude of sulfated mucopolysaccharide synthesis in corneal wounds (32, 33).

In these various studies the sites of labeled precursor incorporation into a specific biochemical synthesis was determined by autoradiography using either stripping film or nuclear emulsion dip techniques. The latter techniques were chosen to evaluate the beta radiation effects on RNA, DNA, and sulfated mucopolysaccharides biosynthesis in the healing cornea.

Methods

Tritiated uridine uptake was selected for studies of incorporation into nuclear RNA synthesis. Tritiated thymidine uptake was used to determine DNA synthesis in cells involved in corneal repair. Sulfur-35-labeled sulfate was used to locate sulfated mucopolysaccharide synthesis in the corneal wound. In addition, numbers of cells carrying on nucleic acid synthesis were tabulated, grain counts were made to determine the relative amounts of nucleic acid synthesis per cell, and microphotometric methods were used to determine the relative amounts of DNA in the nuclei of the various cells involved in the repair process. These data were compared for non-irradiated and irradiated corneal wounds in separate eyes in the same animal and the statistical significance of any difference between control and irradiated eyes was determined.

Autoradiography

Forty-five (45) albino rabbits were used in this study. Fifteen (15) had received 10,000 rads of beta radiation immediately after surgical wounding, 20 had received the same dose two and one-half months before wounding, and 10 had received 10,000 rads of beta radiation ten months before wounding.

The stages of healing studied were 21, 24, 48, 72, and 96 hours and 5, 7, 10, 11, and 20 days after surgical wounding. One hour prior to sacrifice, each animal was anesthetized with sodium pentobarbital intramuscularly, 2 to 3 drops of topical 0.5 percent proparacaine hydrochloride was administered, the eye was exposed with standard lid retractors and a right angle, self-closing entry to the anterior chamber through the cornea was made with a 27 gauge needle attached to an empty microliter syringe. In another quadrant of the cornea another right angle entry was made into the anterior chamber with a 27 gauge needle and syringe containing labeled precursor dissolved in Eagle's basal medium. The empty syringe was used to remove approximately 0.1 to 0.2 ml. of aqueous humor, then the other syringe was used to inject 0.05 ml. of labeled precursor into the anterior chamber.

The following radioactive precursors were used, each in 0.05 ml. per separate intracameral injection: 2 µCi. of Thymidine-^{3}H (specific activity 1.2 curies per millimole), 5 µCi. of Uridine-^{3}H (specific activity 4.1 curies per millimole), and 100 µCi. of carrier free $Na_2{}^{35}SO_4$.

After the one-hour labeling time, the animals were sacrificed with an intravenous overdose of sodium pentobarbital. The eyes were enucleated, fixed in 10 percent buffered formalin for 12 to 24 hours, washed in running water for two hours, and embedded in parafin. Eight-micron sections were cut with an AO-microtome. Serial sections were cut and placed on slides previously "subed" in 0.05 percent ammonium chromium sulfate with 0.5 percent glycerin added. The corneas containing ^{35}S were, in addition, soaked for 20 minutes in an 0.1 percent solution of non-radioactive sodium sulfate to remove the unbound ^{35}S label.

In complete darkness the slides were dipped in Kodak NTB-2 Nuclear Emulsion which had been gradually heated to a smooth liquid (about 40° C.). The Kodak NTB-2 Nuclear Emulsion has a spectral sensitivity particularly suited for autoradiography with tritium labeled compounds and those labeled with sulfur-35. After dipping, the slides were placed on end in racks to dry, sealed in light-tight boxes with dessicant vials, and stored at 10° C. for four weeks, ten weeks, six months, or eight months. Included in each box was one blank slide, dipped in the photographic emulsion and exposed briefly to a strong light source. When developed, this slide served as a control to determine the extent of latent image fading, if any, that might have occurred during the exposure period.

Developing was carried out using Kodak Tri-Chem Pack solutions for 2 minutes developing time in Kodak Dektol developer at 17° C. with constant agitation at 22 movements per 15 seconds, 30 seconds

in Kodak Universal Stop, 8 minutes in Kodak Universal Fixer, and 2 hours wash in running water. Staining in hematoxylin and eosin proved to be unsatisfactory as the eosin destroyed the photographic emulsion. Metanil yellow was finally determined to be a good cytoplasmic counterstain for hematoxylin staining of the developed slides. Normal dehydration to xylene was not possible as the xylene caused fading and complete removal of the metanil yellow, hence a glycerol mounting medium was used for coverslipping.

Labeled cell counts

The numbers of cells labeled with either tritiated thymidine or tritiated uridine were counted within one field width (approximately 350 mμ wide at x400 magnification) of the wound edge. Identical areas were counted on replicate sections and the standard deviation among sections of the same eye was calculated. The mean labeled cell count for irradiated corneal wound was compared with the mean count from the contralateral control eye. The labeled cell count means for all eyes enucleated at the same time were also grouped according to irradiated vs. non-irradiated controls. Comparisons of the group mean labeled cell counts were made and the standard deviations of the group mean labeled cell counts were calculated.

Grain counts per cell

The relative amounts of RNA synthesis occurring during the one-hour labeling time were determined by comparing silver grain counts per cell. Twenty (20) cells from each cell layer of the corneal wound were examined under oil immersion (x1000) and numbers

of silver grains overlying each synthesizing cell were recorded. Replicate slides were counted and the mean grain count and standard deviation per cell layer per cornea were compared between irradiated and non-irradiated corneas for each animal. These comparisons were used to estimate the relative differences in the rates of DNA or RNA synthesis among different cells in different layers of the wounded cornea.

Grain counts were not feasible for the normal cells labeled with tritiated thymidine as the silver grains overlying each DNA synthesizing cell were so numerous that resolution among grains was impossible even after only four weeks exposure to the tritium emissions (Fig. 60). There were discernable differences in DNA synthesis between the irradiated and non-irradiated controls; however, these would have required different techniques to quantify.

Determinations of DNA content per nuclei

Since the relative amounts of DNA synthesis per one hour labeling time could not be determined by grain counting procedures, the total relative DNA content per cell nuclei was measured by microphotometric histochemical techniques.

Photometric measurements of nuclear DNA within cells have been made using the histochemical Feulgen reaction which specifically stains DNA (113). The specificity of the Feulgen reaction stain for the DNA molecule has been confirmed by DNA ultraviolet measurements (114). The photometric measurements of Stowell (113) used a slightly modified Feulgen reaction technique to stain DNA within cell

nuclei. Visible light was filtered through a green gelatin filter and passed through the stained cells under high magnification. The difference in light transmitted through stained and unstained tissue was compared and the mean percent absorption due to the stain was calculated.

In the following experiments a modified version of Stowell's classic technique was used. Ten sections, eight microns thick, of each eye were carefully cut with a Porter-Blume microtome and placed on precleaned slides. All sections were simultaneously stained by a standard Feulgen reaction technique (115) with a carefully controlled acid hydrolysis step. No counterstain was used and the sections were mounted in Permount.

Feulgen stain absorption of visible light was measured using a modified microscope eyepiece containing a fiber optics sensor probe designed to be in focus simultaneously with the viewed image. The sensor tip of the probe is small enough (0.15 mm. in diameter) to be positioned over a small portion of a cell nucleus under oil immersion magnification (x1000).

The light from the monitored area is transmitted from the fiber optics bundle in the eyepiece to a larger fiber optics bundle through a green barrier filter that passes light between 5000 Å and 5400 Å only and, hence, to a sensitive photomultiplier tube. The photomultiplier tube (EMI 9502 gain of 29×10^6 at 1500 V.) output signal is fed into a solid state photometer capable of giving full scale deflection for a current input of less than 2 nanoamps. The photo-

multiplier tube and photometer have been used in slip lamp fluorophotometry as described by Waltman and Kaufman (116). The photometer output was recorded on a chart recorder giving continuous measurements as the eyepiece probe was moved over the stained sections.

Photometric measurements were made of the light transmitted through the slide, mounting media, and coverslip to establish baseline sensitivity adjustments on the chart recorder and provide a control measurement for the maximum light intensity transmitted through the slide with no tissue absorption. The probe was then moved over an area of cytoplasm of a particular cell to determine the absorption of the unstained cytoplasm. The eyepiece probe was then moved over different parts of the Feulgen stained cell nucleus while continuous measurements were being recorded.

Twenty cell nuclei each of epithelial cells and stromal cells involved in the corneal wound were measured per histological section. Replicate sections were measured. The recorded measurements were tabulated and the percent absorption due to the DNA specific Feulgen stain was calculated from the difference between the maximum intensity transmitted by the tissue without stain (I_{max}) and the maximum intensity transmitted through the stained DNA within the nucleus (I_{nuc}) according to

$$\text{Mean percent absorption} = \frac{\text{mean } I_{max} - \text{mean } I_{nuc}}{\text{mean } I_{max}} \times 100 \ .$$

The mean percent absorption was compared for the irradiated corneal cells and the contralateral, non-irradiated corneal cells and the statistical significance of the differences was calculated.

Results

Significant differences in DNA and RNA synthesis were found between cells in the irradiated corneal wounds and cells in the non-irradiated, contralateral control wounds during the first 20 days of healing. Labeled cell counts show that the radiation treatments had produced a severe reduction in the numbers of involved cells that were synthesizing DNA and RNA, especially during peak synthesis activity at approximately four and five days after wounding. The beta radiation effect of reducing the numbers of cells synthesizing nucleic acids was greatest in those corneas that were irradiated immediately after surgery, less in those corneas that had been irradiated two and one-half months before surgery, and least in those that had been irradiated ten months before surgery. Quantitative measurements of the DNA content per cell and the grain counts for RNA synthesis both indicate that the beta radiation treatments had reduced the levels of DNA and RNA synthesis per cell, in addition to reducing the numbers of cells that were synthesizing nucleic acids. Mucopolysaccharide synthesis in the healing cornea was also affected by the beta radiation treatments. There was a discernable difference in mucopolysaccharide (MPS) synthesis, as indicated by bound ^{35}S incorporation in the wound area, between the irradiated and the non-

irradiated corneas. Quantification of this difference in MPS synthesis was not possible with the techniques available. The detailed description of these results appears below.

Labeled cell distribution

In the non-irradiated corneal wounds, DNA synthesis was apparent within 24 hours after wounding. The numbers of labeled cells observed in the wound area and in the periphery increased to a maximum by four to five days as described previously by Bracher (32). The distribution of labeled cells found during the first 20 days of wounding is described below.

Several fibroblasts and keratocytes were found labeled near the wound edge at 24 hours of corneal healing. There were more labeled cells in the posterior stroma than in the anterior portion. No epithelial cells were found labeled except for a few isolated epithelial cells at the limbus. By 48 hours, more labeled fibroblasts were observed along the wound edge and more keratocytes were synthesizing DNA in the general wound area of the stroma. Several epithelial cells were found heavily labeled within 1 to 2 mm. of the wound edge. Directly below these epithelial cells were several heavily labeled fibroblasts apparently migrating toward the wound. No endothelial cells were found labeled with the thymidine-^{3}H precursor.

After three days of healing, more epithelial cells were labeled, particularly the basal epithelial cells. It seemed that the majority of basal epithelial cells of the entire central half on the non-

irradiated cornea had begun to synthesize DNA almost simultaneously, sometime between two and three days of healing. Among the more numerous labeled fibroblasts found in the general wound area, a few were noted in the posterior wound amid the fibrin filaments of the aqueous clot that filled the wound posteriorly. By four days more, DNA synthesizing fibroblasts had lined up along the axis of the incision and more peripheral keratocytes were found labeled throughout the thickness of the stroma. Epithelial cell DNA synthesis was still quite extensive, especially in those cells involved in the wound and basal epithelial cells peripheral to the wound. After five days of healing, the extent of DNA synthesis began to decrease in all layers of the wound and adjacent areas. By 20 days, only a few basal epithelial cells and several fibroblasts involved in the wound were labeled.

In the corneas that were irradiated immediately after wounding, the number of cells incorporating thymidine-^{3}H was markedly reduced during the observation period. By 48 to 72 hours, only a few labeled keratocytes were found in the entire stroma. These were located in the posterior half of the stroma adjacent to the wound edge. Only a few epithelial cells in the wound were synthesizing DNA. Those cells that were labeled seemed to have less silver grains overlying them; however, the grain density of the non-irradiated corneal cells at this time was too great for a numerical comparison. The thymidine-^{3}H labeled cell distribution was approximately the same throughout 20 days of healing.

The same labeling pattern was found in those corneas that had been irradiated two and one-half months before wounding. More labeled cells were found in the peripheral stroma in these corneas than in those that had been irradiated immediately after wounding. The prominent lack of epithelial cell DNA synthesis was found characteristic of these irradiated corneas throughout day 20. By this time several labeled fibroblasts and keratocytes were found in the anterior third of the stroma. In some irradiated corneas, after 20 days of healing there were numbers of DNA synthesizing cells comparable to those found in the non-irradiated corneas at this time.

The distribution of DNA synthesizing cells in the corneas that had been irradiated ten months prior to wounding was more normal than that observed in the corneas irradiated either two and one-half months before or immediately after surgery. Although fewer labeled cells were found in the irradiated corneas than in the non-irradiated corneas during the 20-day healing period, the beta radiation inhibition of DNA synthesis was not as dramatic as in the other irradiated groups.

Labeled cell counts -- DNA

In the corneas irradiated immediately after surgery the number of cells synthesizing DNA averaged only 11 percent of the number of cells synthesizing DNA in the contralateral, non-irradiated corneal wounds during the periods of peak synthesis ($p < 0.005$), as illustrated in Fig. 62. In the corneas irradiated two and one-half months before

TABLE 4

LABELED CELL COUNTS FOR DNA SYNTHESIS

Treatment		Healing Time After Surgery (Days)						
		1	2	3	4	5	10	20
Irradiated at Surgery								
Non-irradiated	Mean	28.9	35.8	36.4	68.5	66.6	—	16.3
	S.D.	19.6	27.5	6.1	13.4	2.8	—	6.0
Irradiated	Mean	0.3	0.4	2.7	3.9	5.2	—	6.4
	S.D.	0.2	0.2	2.4	1.8	3.2	—	6.1
Irradiated Two and One-Half Months Prior to Surgery								
Non-irradiated	Mean	21.5	38.3	29.8	60.5	—	36.4	—
	S.D.	2.1	4.5	2.9	4.9	—	6.1	—
Irradiated	Mean	13.5	12.7	17.3	22.0	—	20.2	—
	S.D.	2.1	4.5	12.4	3.8	—	4.8	—
Irradiated Ten Months Prior to Surgery								
Non-irradiated	Mean	26.2	37.6	40.2	—	62.4	—	19.8
	S.D.	4.4	6.9	5.1	—	4.9	—	5.3
Irradiated	Mean	16.1	—	24.3	32.4	38.3	—	15.2
	S.D.	4.9	—	5.4	4.2	6.1	—	4.5

surgery the mean labeled cell count was 35 percent of the non-irradiated controls ($p = 0.01$) during peak synthesis at four days of healing (Fig. 62).

Table 4 shows that the reduced number of labeled cells in the corneas irradiated ten months prior to wounding were comparable at day 1 to those numbers of cells synthesizing DNA in the corneas irradiated two and one-half months before wounding. The extent of the radiation inhibition of cells synthesizing DNA was not as great at peak synthesis, however. The number of labeled cells in the ten month pre-irradiated corneas was 61 percent of the number found labeled in the non-irradiated corneas ($p = 0.05$). By 20 days of healing the number of labeled cells observed in the ten months pre-irradiated corneas was almost the same as the number observed in the non-irradiated corneas. Apparently, the ten month period following the beta radiation treatments was sufficient time for partial recovery of the DNA synthetic capability of many cells that were later involved in healing after surgery.

DNA content per nucleus

The microphotometric measurements of Feulgen stained DNA in epithelial and stromal cell nuclei were carried out on corneal samples after four and five days of healing, when the number of synthesizing cells was maximum.

The beta radiation treatments were responsible for a significant reduction in the average DNA content of epithelial and stromal cell nuclei in the healing cornea. In the corneas that were irradiated

TABLE 5

RELATIVE NUCLEAR DNA CONTENT IN HEALING CORNEA

	Relative Absorption*		Decrease in DNA Per Cell (%)	Probability of Error (p)
	Non-irradiated	Irradiated		
Irradiated at Surgery				
Epithelial	39.6	26.7	32.5	0.05
Stromal	43.4	30.3	30.2	0.01
Irradiated Two and One-half Months Prior to Surgery				
Epithelial	47.5	34.5	27.3	0.005
Stromal	33.3	24.6	26.1	0.04
Irradiated Ten Months Prior to Surgery				
Epithelial	37.3	31.4	15.7	0.05
Stromal	41.7	34.6	17.0	0.02

*Relative absorption figures based on microphotometric measurements of light intensity transmitted through cell nucleus stained by Feulgen reaction.

immediately after surgery the DNA content of the epithelial nuclei and the stromal cell nuclei was 32.5 percent and 30.3 percent less, respectively than the DNA content measured in the non-irradiated controls. The effect on the amount of DNA per nucleus was not as great in those corneas irradiated two and one-half months before wounding (Table 5). If ten months recovery time was allowed between irradiation and wounding, the DNA content per nucleus was only decreased by 15.7 percent in the epithelial cells and by 17.0 percent in the stromal cells of the irradiated corneas.

Labeled cell distribution -- RNA

In the non-irradiated control corneas RNA synthesis was well established in the basal epithelial cells near the wound at 24 hours of healing. At this time only a few isolated keratocytes were found labeled with uridine-^{3}H. By 48 hours, epithelial cells were labeled from the wound edge, peripherally , approximately one-half the distance to the limbus. Several keratocytes and fibroblasts were synthesizing RNA in the immediate wound area. Moving outward from the wound edge the number of cells and the extent of label uptake per cell diminished rapidly. The majority of the labeled cells were found in either the anterior third or the posterior third of the central stroma. Endothelial RNA synthesis was apparent in the central half of the endothelial layer. Peripheral endothelial cells, near the limbus, were not labeled. Fibroblasts were the heaviest labeled cells observed at 48 hours of healing.

TABLE 6

LABELED CELL COUNTS FOR RNA SYNTHESIS

Treatment		Healing Time After Surgery (Days)						
		1	2	3	4	5	10	20
Irradiated at Surgery								
Non-irradiated	Mean	—	149.0	256.0	—	393.0	156.0	101.0
	S.D.	—	3.2	36.7	—	25.4	28.9	8.1
Irradiated	Mean	—	76.3	60.0	72.1	—	65.0	50.3
	S.D.	—	7.7	14.2	1.24	—	11.3	4.8
Irradiated Two and One-Half Months Prior to Surgery								
Non-irradiated	Mean	71.3	165.7	—	411.2	—	161.4	98.1
	S.D.	7.5	6.7	—	11.3	—	9.2	7.7
Irradiated	Mean	29.5	122.0	—	142.0	—	125.0	131.3
	S.D.	2.1	11.5	—	5.2	—	14.6	11.1
Irradiated Ten Months Prior to Surgery								
Non-irradiated	Mean	—	172.9	—	—	455.0	—	—
	S.D.	—	8.1	—	—	19.8	—	—
Irradiated	Mean	—	155.3	—	—	348.0	148.7	164.1
	S.D.	—	9.9	—	—	13.4	17.5	11.8

After 72 hours, most of the epithelial cells were heavily labeled in the central half of the cornea. Endothelial cells and peripheral keratocytes were labeled less than central keratocytes and fibroblasts. Scattered active keratocytes and fibroblasts were found adjacent to the wound area, seemingly migrating toward the wound. By day 4, epithelial RNA synthesis was widespread throughout the entire epithelial layer, from limbus to the wound. RNA synthesis was especially prominent in the basal epithelial cells nearest the wound. Labeled endothelial cells were found from the wound edge, peripherally, almost to the limbus. The heaviest labeling was observed in the fibroblasts and keratocytes found within the wound and 1 to 2 mm. on either side of the wound perimeter. Moving outward, the cells seemed to be labeled less, and fewer numbers of labeled cells were found in the stroma. Peak RNA synthesis was apparent in the four- and five-day old wounds. Thereafter, the frequency and extent of label uptake decreased steadily. By 20 days the RNA synthesis in the epithelium and stromal keratocytes was minimal. Endothelial cell label was significant, often greater than the epithelial cells at this time. Fibroblasts found along the wound edge were few in number and only moderately labeled at the end of the observation period.

In the irradiated corneas the RNA synthesis was dramatically reduced in both the number of synthesizing cells and the extent of synthesis per cell. In the corneas irradiated immediately after or two and one-half months prior to surgery, the first uridine-^{3}H

uptake was detectable in some eyes enucleated after 48 hours of healing. The epithelial cells were then beginning to synthesize RNA and the extent of label per cell was minimal. A few fibroblasts and keratocytes along the stromal portion of the wound were lightly labeled; however, no endothelial cells were synthesizing RNA. The radiation inhibition of RNA synthesis continued throughout the third and fourth days of healing. No detectable increase in uridine-^{3}H uptake was noted in any of the different cells during this time. The labeled cells in the four-day irradiated wounds were scattered and substantially less numerous than the labeled cells in the controls. The keratocytes, fibroblasts, and epithelial cells in the irradiated corneas were more heavily labeled than the endothelial cells in the same corneas. Sections examined at 10 and 20 days after wounding showed the same pattern of RNA synthetic inhibition among all cells of the irradiated corneas. Even after 20 days of healing the number and the rate of synthesizing cells of RNA synthesis in the irradiated corneas never approximated the synthesis found in the non-irradiated controls.

In the corneas irradiated ten months before surgery the radiation inhibition of RNA synthesis was not as pronounced. After two days of wounding less inhibition was noted in the corneas irradiated ten months before wounding than was observed in the corneas irradiated two and one-half months prior to wounding. By five days the numbers of cells incorporating uridine-^{3}H in the irradiated

corneas was only slightly less than the non-irradiated controls. After 20 days the irradiated corneas appeared to have more cells synthesizing RNA than the controls. The tabulation of the number of labeled cells found per unit area illustrates these differences (Table 6).

RNA-labeled cell counts and grain counts

The number of labeled cells found in the wound area at the times of peak RNA synthesis, i.e., four and five days of healing, reflected the magnitude of the radiation inhibition of uridine-^{3}H uptake. Earlier, at two days of healing, in those corneas irradiated at the time of surgery, the number of labeled cells was reduced by 49 percent, as compared to the controls (Table 6). In the corneas irradiated earlier (two and one-half months before wounding) the number of RNA-synthesizing cells was reduced some 26 percent below the numbers found in the contralateral control corneas. The radiation inhibition was even less in the corneas irradiated ten months prior to wounding.

The overall inhibition of uridine-^{3}H uptake is illustrated in Figure 72 for those corneas that were irradiated two and one-half months before wounding.

In the irradiated corneas at day 4, the number of cells synthesizing RNA was only 34 percent of those labeled cells found in the non-irradiated control corneas. At the same time the amount of RNA synthesized per cell was almost three times greater in the

controls than in the irradiated corneas. The mean grain count for the control cell was 31 grains per cell while the mean grain count for the cells in the irradiated cornea was 11 grains per cell. Paired sample statistics showed a high significance for this difference ($p < 0.01$).

A trend was noted in the corneas after periods of peak RNA synthesis. The cells synthesizing RNA in the control corneas diminished in numbers much faster than did the numbers of cells in the irradiated corneas. By 20 days of healing the labeled cells in the two groups of corneas that were pre-irradiated before surgery were more numerous than the labeled cells in the non-irradiated corneas.

Sulfur-35 Uptake (Mucopolysaccharide Synthesis)

Bound radioactive sulfate was found localized along the wound edge of the non-irradiated control cornea by five days of healing. Label was found scattered over the entire cornea, but a higher concentration of radioactive sulfate was observed in the anterior stroma along the wound. By the tenth day the rate of sulfated mucopolysaccharide (MPS) synthesis was definitely increased all along the wound margin. The incorporation of labeled sulfate was correlated with the concentration of fibroblasts and keratocytes in the immediate wound area. The synthesis of sulfated MPS was roughly confined to the area of new connective tissue formation, indexed by the lack of lamellar architecture found in newly synthesized

stromal tissue. After 20 days of healing the labeled sulfate pattern was unchanged from the distribution noted at day 10.

In the irradiated corneas the bound radioactive sulfate was diffusely scattered over the stroma at five days of healing. Only a few sections demonstrated any localization of sulfate incorporation along the wound margin. By comparison the irradiated corneas seemed to have slightly less sulfate turnover than did the non-irradiated corneas. By day 10, labeled sulfate incorporation was localized at the wound edge; however, the concentration was consistently much less than was formed in the control corneas at the same period of healing. The most obvious concentrations were found overlying the few fibroblasts and keratocytes that were involved in the wound (Figure 74).

Although no quantitative evaluations were made of the reduction in sulfated mucopolysaccharide synthesis in the irradiated corneas, it was observed that the number of fibroblasts and keratocytes involved in the wound were less than one-half the numbers found in the control corneal wounds. Subjectively, it seemed that the reduction in bound radioactive sulfate roughly correlated with the reduction in cells involved in the wounds of irradiated corneas. Twenty-day wounds were not noticeably different from 10 day wounds in the irradiated corneas.

Comment

The substantial decrease in synthesis of sulfated mucopolysaccharides, indexed by a decrease in ^{35}S-labeled sulfate incorporation in the wound area of the irradiated corneas, was not surprising. Labeled sulfate uptake in corneal wounds has been previously correlated with the connective tissue cells and fibroblasts directly involved in wound repair (33). The same correlation was noted in the non-irradiated control wounds just described above. The decrease in the numbers of cells observed in the wounds of irradiated corneas would probably be enough to cause a noteable decrease in sulfated mucopolysaccharide synthesis, even if the cells were fully capable of normal rates of synthesis. The observed ultrastructural changes in these involved cells, described in Section 4, would indicate that the cells were not fully competent to synthsize new connective tissue, particularly collagen. This incompetence is probably not a manifestation of a specific biosynthetic blockage, but rather, it seems to be an overall inability of the irradiated cell to accept the responsibility of synthesizing new connective tissue substances to be used extracellularly in reconstruction of the damaged tissue within the wound. Extending this theme one would expect that sulfated mucopolysaccharide synthesis, like collagen, DNA, and RNA synthesis, might be inhibited as a result of the beta radiation treatments.

It is interesting that the magnitude of the inhibition of DNA and RNA synthesis during corneal healing was substantially diminished when a long recovery period followed the beta radiation treatments.

The longer the elapsed time between irradiation and surgical wounding, the smaller was the observed reduction of numbers of synthesizing cells and the reduction of DNA synthesis per cell. It is possible that recovery begins immediately after the radiation damage, however, the recovery process must be relatively slow in terms of epithelial function, yet reasonable in terms of stromal cell turnover, if repopulation is the basis for recovery from the radiation damage. It would seem that the reappearance of synthetically competent cells is not necessarily a linear function of the elapsed recovery time. Indeed, if the recovery was based on repopulation of irradiated, incapacitated cells at normal cell turnover rates, the observed recovery should be a decreasing exponential function of time. Unfortunately, not enough data were available for different times of irradiation before surgery to establish the exact time relationship of the observed recovery of the cells' ability to synthesize nucleic acids following wounding. The data available would fit such a scheme, however.

Comparing the relative synthetic capabilities of the corneal cells irradiated immediately after surgery, two and one-half months prior, and ten months prior to surgery (Figs. 61, 62, and 63), it should be noted that the ten month recovery time resulted in the reappearance of the normal rise and fall pattern of DNA synthesis during the first 20 days of healing. This pattern was not obvious in the corneas irradiated two and one-half months before wounding.

The relative magnitude of radiation inhibition of the number of cells synthesizing DNA was approximately the same as the inhibition of cells synthesizing RNA at the times of peak synthesis. This is illustrated by the three-fold decrease in number of cells synthesizing DNA and RNA in those corneas that were irradiated two and one-half months before wounding (Figs. 63 and 73). The synthesis per cell was not comparable at this time, however, as the DNA content per cell was only reduced 30 percent below normal, while the rate of RNA synthesis was reduced by 65 percent from that of the non-irradiated control corneas.

The characteristics of the beta radiation inhibition of DNA synthesis are important to the delineation of the possible mechanisms that might be responsible for the observed inhibition. It has already been noted that the presynthesis or G_1 period of interphase is more radiosensitive than the synthetic (S) period (107). The observed inhibition of DNA synthesis resulting from irradiation during the postsynthetic or G_2 period is considered by many to be a result of inhibition of mitosis, thereby causing a lack of initiation of DNA synthesis in the succeeding cycle (117).

Inhibition of DNA synthesis resulting from a G_2-effect is characterized by a greater inhibition of the number of synthesizing cells and a smaller inhibition of the rate of DNA synthesis in individual cells. The same dichotomy was noted in the beta radiation inhibition of DNA synthesis previously described. It appears that the beta radiation inhibited DNA synthesis in the healing corneas

through a G_2-effect, however, this is not the only possible mechanism. Inhibition of DNA synthesis by irradiation during the G_1 period of interphase has been explained on the basis of radiation induced changes which affect the formation of the template necessary for DNA replication (118). This mechanism would also fit with the observed beta radiation inhibition of the number of DNA synthesizing cells and the decrease in DNA content per cell. A G_1-effect would not be inconsistent with the observed long-term inhibitory effect, especially for connective tissue cells, since their turnover in the cornea is thought to be quite slow.

Even though a reduction in DNA synthesis should not be considered as a true index of mitotic inhibition, it was noted in the previous experiments that the beta radiation caused a reduction in the number of connective tissue cells and fibroblasts involved in the wound. If a G_1-effect was the mechanism of beta radiation inhibition of nucleic acid synthesis, then any mitotic inhibition that resulted from a quantitative lack of premitotic DNA would be reflected in the total number of cells involved in the wound at the early stages of corneal healing (before invading cells, monocytes especially, could reach the wound and transform into active fibroblasts). If this were the situation, the presence of a G_1-effect would not exclude the possibility of a G_2 inhibition resulting from blocked mitosis which might become evident at a later time.

The correlation between beta radiation inhibition of DNA and RNA synthesis, simultaneously, may be important in the normal role that DNA plays in the synthesis of RNA. Apparently, DNA serves as a template for RNA synthesis (119). Therefore, the observed decrease in RNA synthesis following wounding of corneas irradiated with beta radiation may reflect some damage to the DNA molecule itself, rather than interference with DNA synthesis.

Figure 54. Autoradiograph of Thymidine-^{3}H labeled cells in non-irradiated wound after 68 hours of healing (DNA). Fibroblasts are heavily labeled at this time. Epithelium (center right) has grown into incision. Fibrin clot extends from center of wound posteriorly (left). (x 250)

Figure 55. Autoradiograph of 68-hour wound in irradiated cornea (DNA). Epithelium has grown into incision but no labeled cells are present. (x 250)

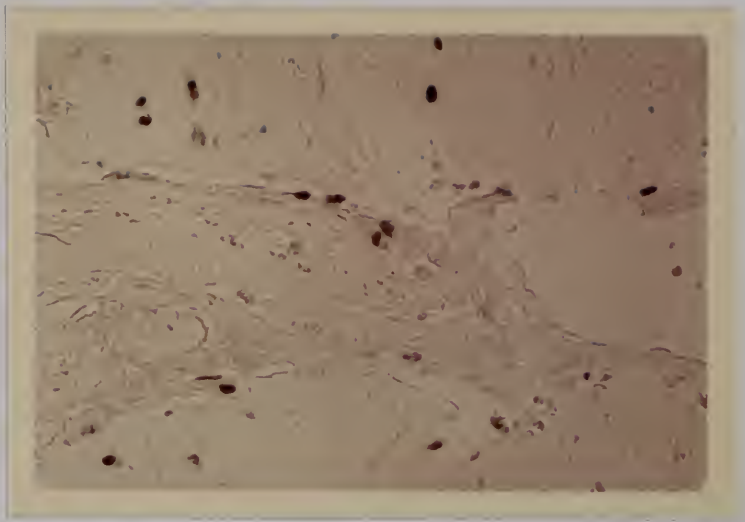
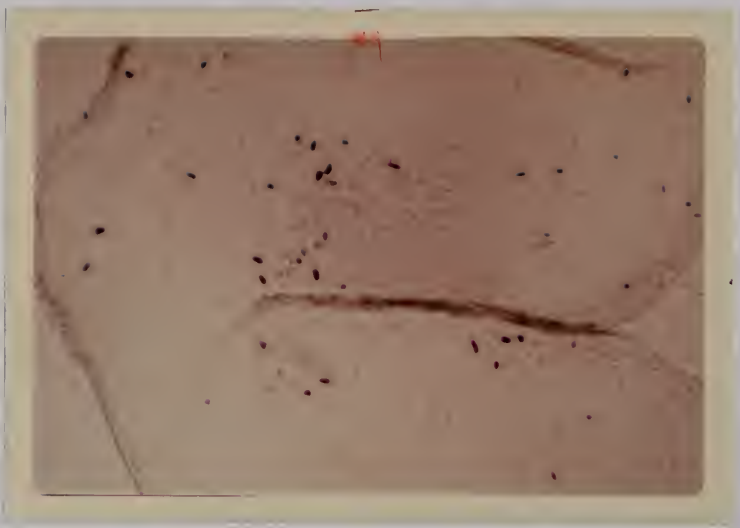


Figure 56. Autoradiograph of Thymidine-^{3}H labeled cells in non-irradiated wound after 91 hours of healing (DNA). Epithelium (right) has grown into incision (center) and labeled cells are concentrating at wound margin. (x 100)

Figure 57. Autoradiograph of 91 hour wound in irradiated cornea (DNA). No labeled cells are found at this time at wound margin (upper left), in epithelium(right), or in adjacent stroma. (x 250)



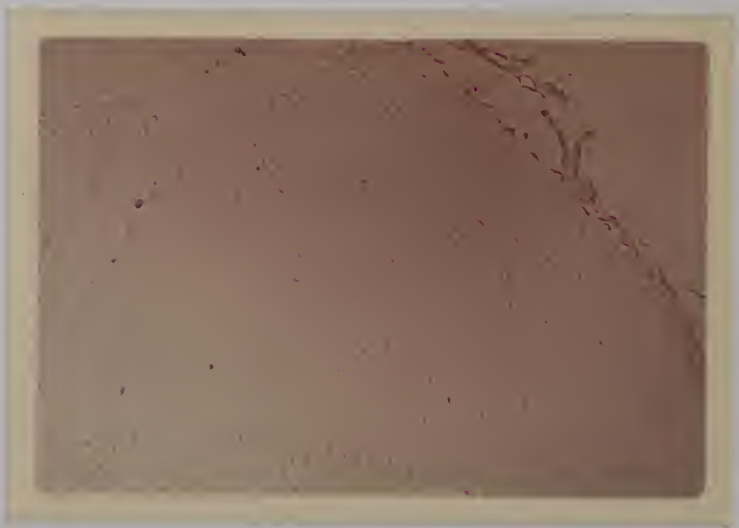
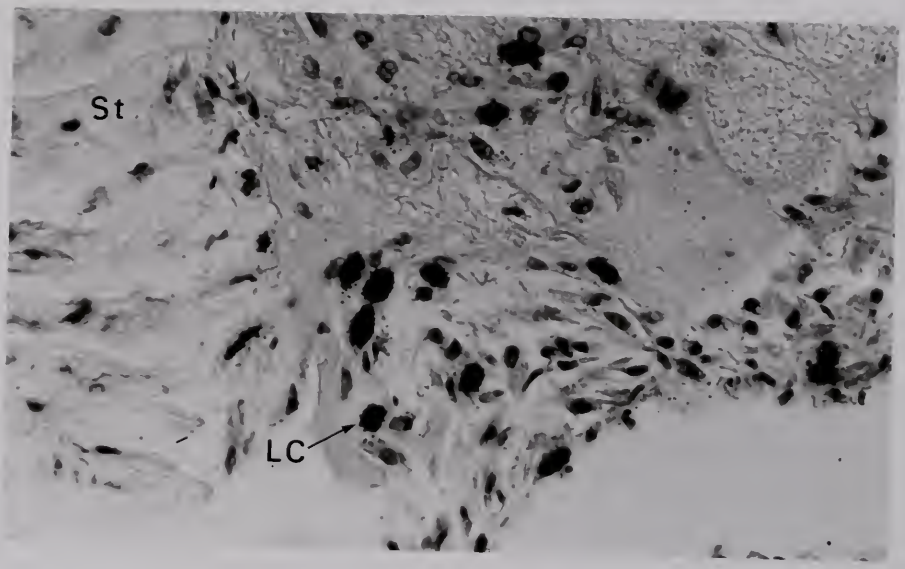


Figure 58. Autoradiograph of DNA synthesis in 120 hour control wound. View shown is of anterior wound. Epithelium (right) and stromal margin contain heavily labeled cells. DNA synthesis is maximum at this time. (x 400)

Figure 59. Autoradiograph of DNA synthesis in 120 hour irradiated wound. Anterior wound with epithelium on the left contains no labeled cells. (x 400)


St
LC

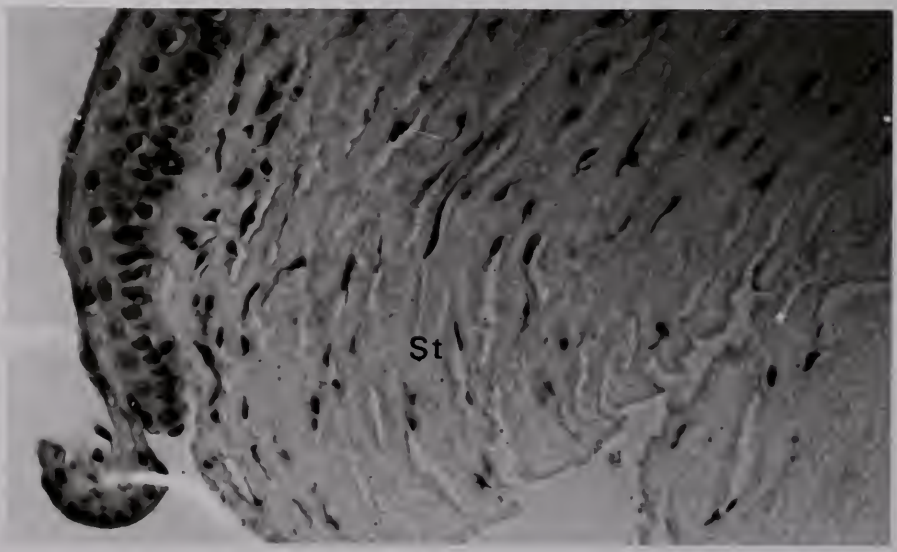

St

Figure 60. Autoradiograph of Thymidine-^{3}H uptake in corneal epithelium at 96 hours of healing. DNA synthesis is extensive in those cells that are incorporating Thymidine-^{3}H precursor. (x 1000, control wound)

Figure 61. Autoradiograph of Uridine-^{3}H uptake in corneal epithelium at 96 hours of healing. The fewer grains per cell made grain counts possible for Uridine-^{3}H uptake. (x 1000, control wound)

Figure 62. Graph of labeled cell counts for DNA synthesis in corneas irradiated at surgery. Counts are based on the number of labeled cells per unit area of wound and adjacent cornea, approximately 300 μ wide field at magnification of 400x.

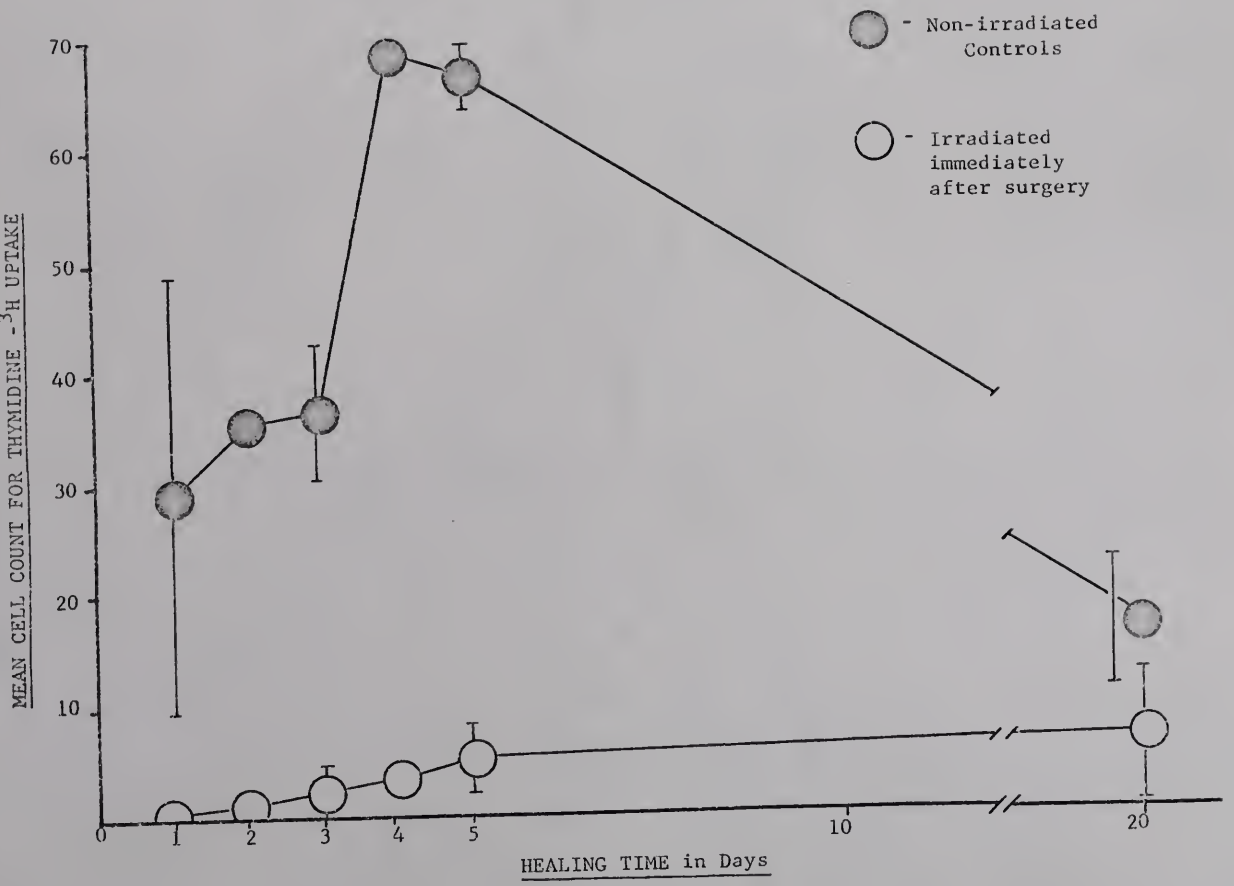

- Non-irradiated Controls
- Irradiated immediately after surgery
MEAN CELL COUNT FOR THYMIDINE - ^{3}H UPTAKE
70
60
50
40
30
20
10
0
1
2
3
4
5
10
20
HEALING TIME in Days

Figure 63. Graph of labeled cell counts for DNA synthesis in corneas irradiated two and one half months before surgery. Figure is based on labeled cell count per unit area of wound. Note partial recovery in irradiated corneas at day three and day four.

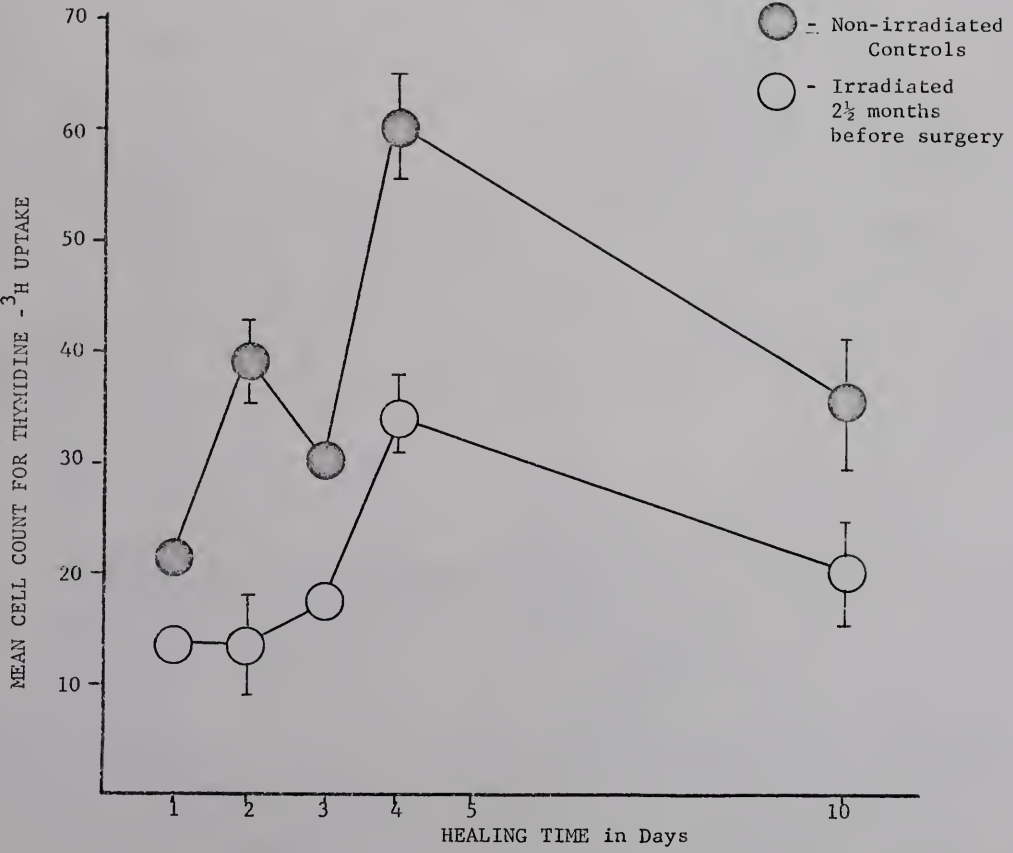

70
60
50
40
30
20
10
MEAN CELL COUNT FOR THYMIDINE - ^{3}H UPTAKE
1
2
3
4
5
10
HEALING TIME in Days
- Non-irradiated Controls
- Irradiated 2½ months before surgery

Figure 64. Graph of labeled cell counts for DNA synthesis in corneas irradiated ten months before surgery. Values are based on labeled cell count per unit area of wound. After ten month recovery time DNA synthesis pattern in irradiated corneas is similar to non-irradiated controls.

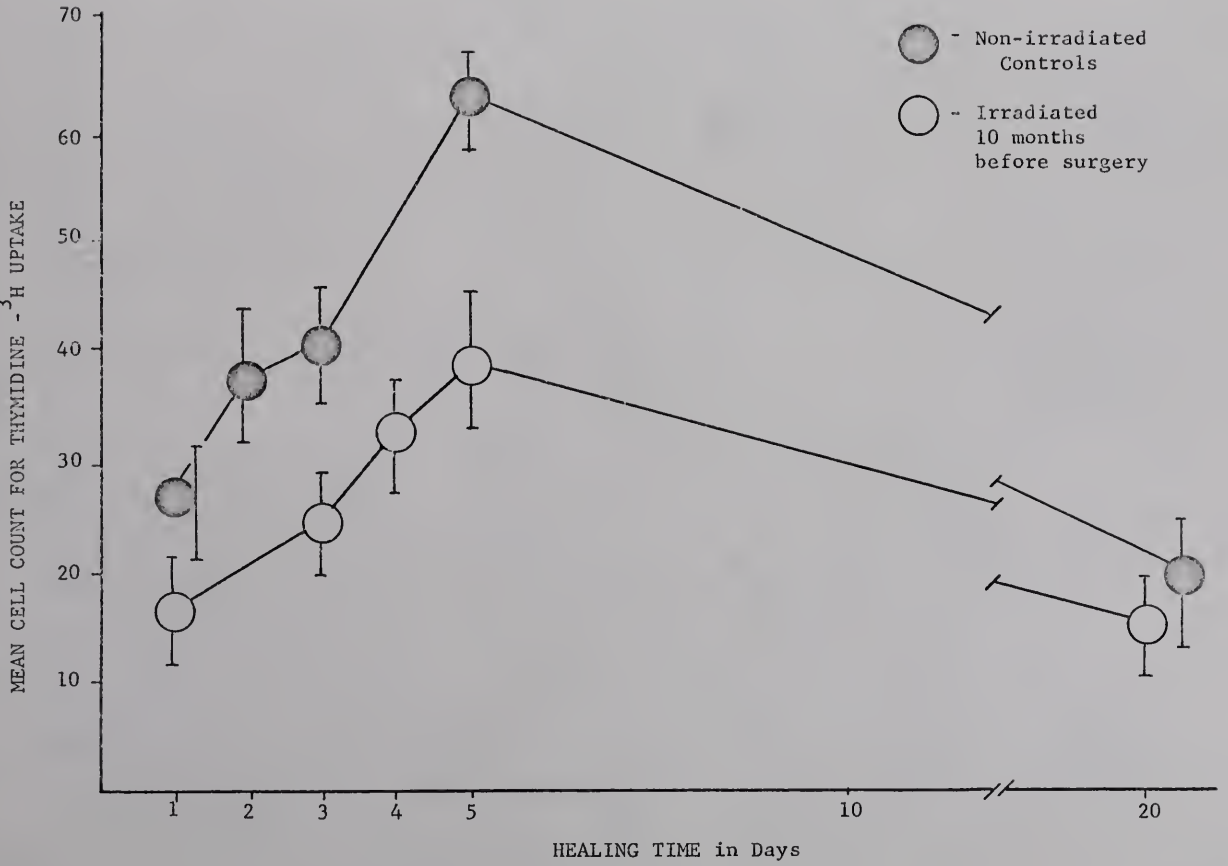
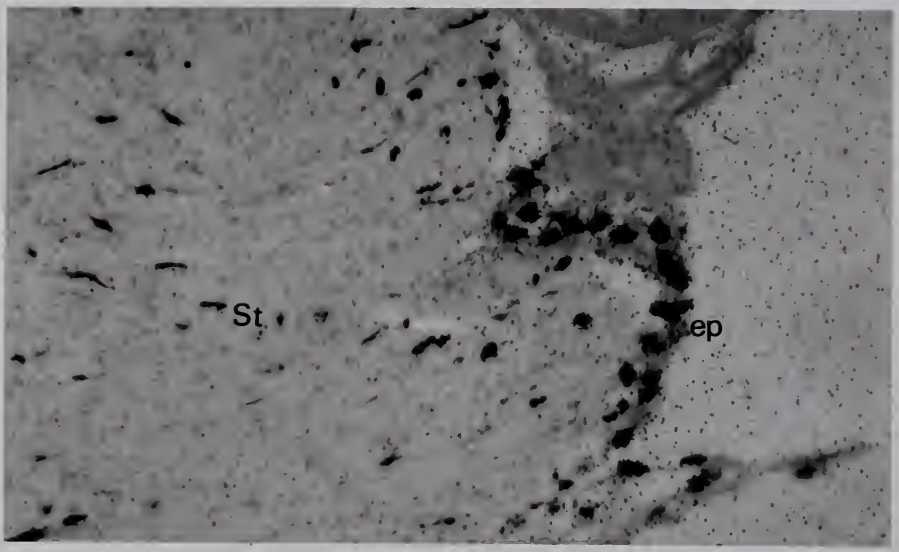

70
60
50
40
30
20
10
MEAN CELL COUNT FOR THYMIDINE - ^{3}H UPTAKE
- Non-irradiated Controls
- Irradiated 10 months before surgery
1
2
3
4
5
10
20
HEALING TIME in Days

Figure 65. Autoradiograph of Uridine-^{3}H uptake in non-irradiated corneal wound after 48 hours of healing. Autoradiograph was exposed six months before developing to identify poorly labeled cells in stroma (St). (x 400)

Figure 66. Autoradiograph of Uridine-^{3}H uptake in irradiated corneal wound after 48 hours of healing. Heavy background identifies autoradiograph as typical of six month exposure before developing. Note decrease in label uptake per cell as compared to controlled cells shown in Figure 65 above. (x 400)


St
ep

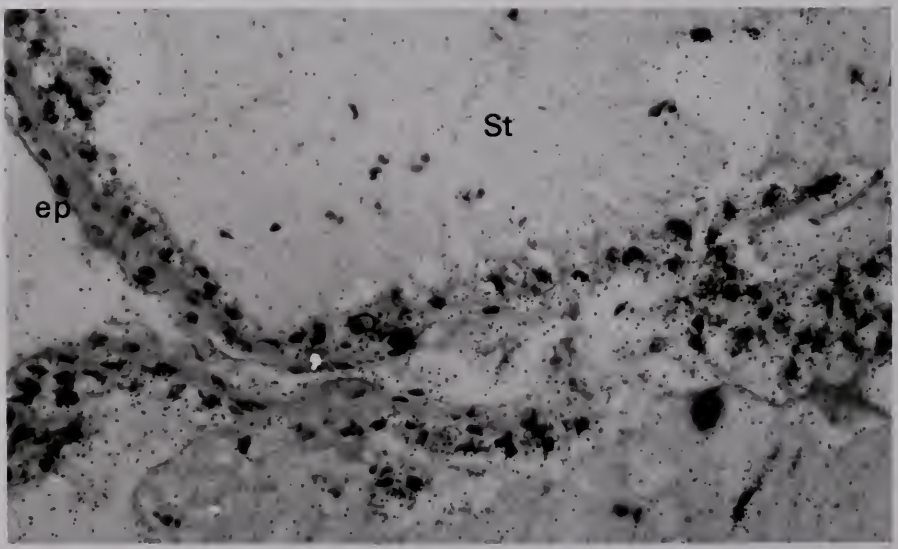
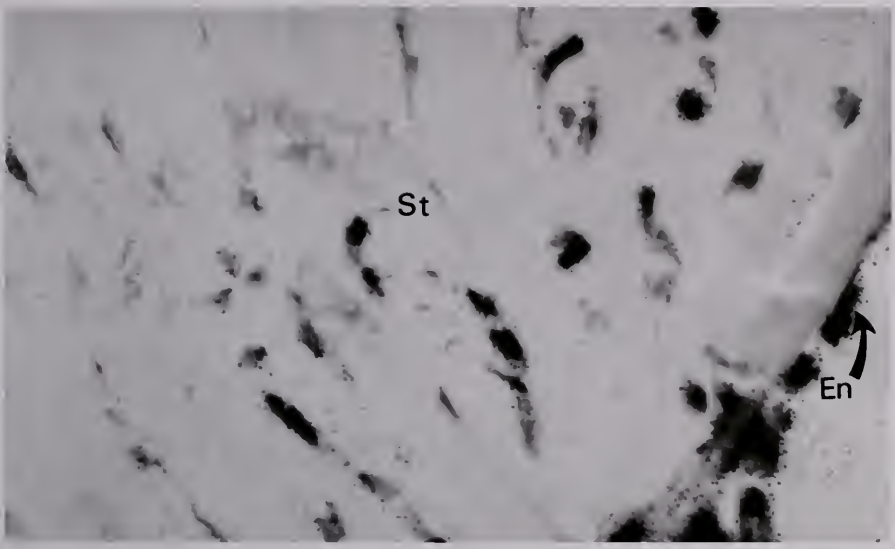

St
ep

Figure 67. Autoradiograph of Uridine-^{3}H uptake in anterior portion of control cornea at 96 hours. Almost all basal epithelial cells (right) are labeled at this time. (x 250)

Figure 68. Autoradiograph of Uridine-^{3}H uptake in anterior portion of irradiated cornea at 96 hours. Only a few basal epithelial cells have taken up RNA precursor. Compared to control cornea shown above in Figure 67 of those few cells that are synthesizing RNA none are incorporating Uridine-^{3}H at a normal rate. (x 250)

Figure 69. Autoradiograph of 96 hour control corneal wound illustrating stromal uptake of Uridine-^{3}H. (x 1,000)

Figure 70. Autoradiograph of control corneal wound illustrating Uridine-^{3}H uptake in endothelium at day 20. (x 1,000)


St
En

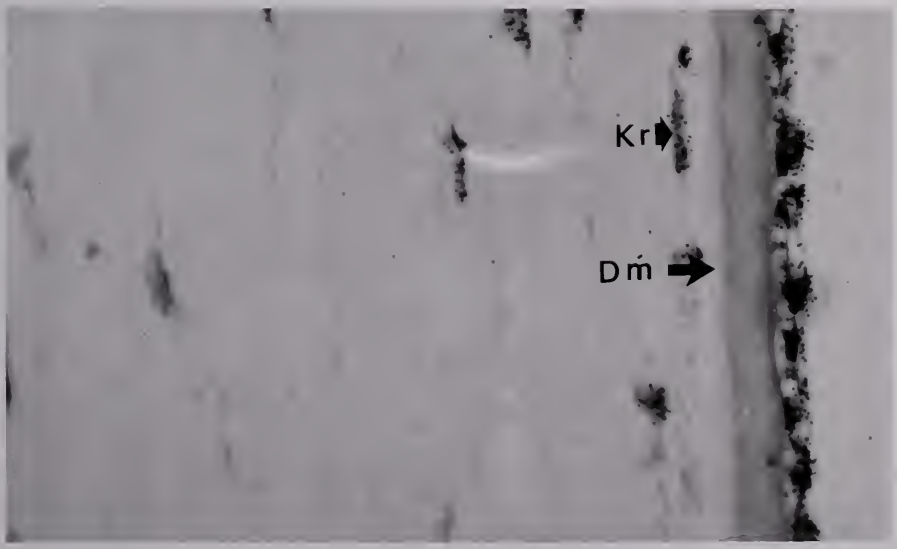

Kr
Dm

Figure 71. Autoradiograph of five-day control wound showing RNA synthesis at endothelium. (x1,000)

Figure 72. Autoradiograph of five-day irradiated wound showing RNA synthesis at endothelium. A notable decrease in the RNA synthesis per cell is obvious compared to non-irradiated endothelial cells (En) and stromal keratocytes (Kr) shown above in Figure 71. (x 1,000)

Figure 73. Graph of labeled cell counts for RNA synthesis in corneas irradiated at two and one half months before surgery. Values are based on labeled cell count per unit wound area. Standard deviations for illustrated values are smaller than circles on graph therefore, not shown in this illustration.

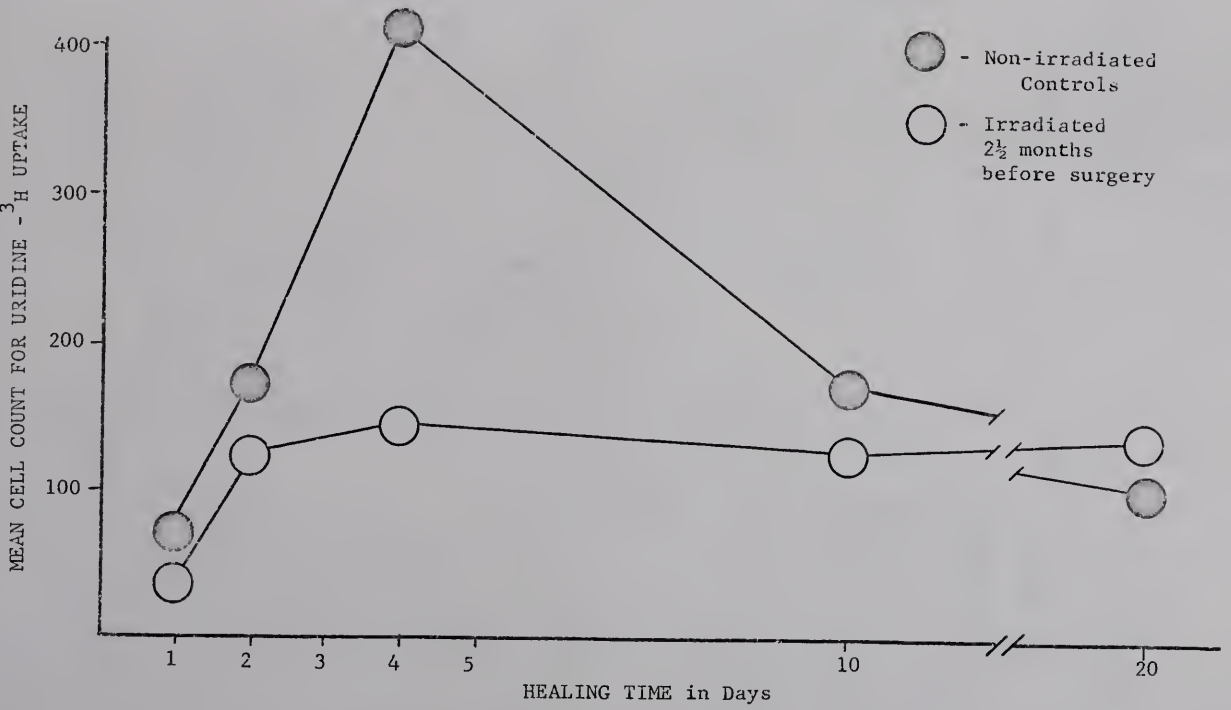

400
300
200
100
MEAN CELL COUNT FOR URIDINE - ^{3}H UPTAKE
- Non-irradiated Controls
- Irradiated 2½ months before surgery
1
2
3
4
5
10
20
HEALING TIME in Days

Figure 74. Autoradiograph of ^{35}S incorporation into sulfated mucopolysaccharides in 20 day wound of non-irradiated cornea. Note concentration of ^{35}S - labeled mucopolysaccharides in stromal wound area (St). Intact areas of stroma (top and bottom) show less mucopolysaccharide synthesis than wound area (center). (x1,250)

Ep
St

SECTION 7

GENERAL DISCUSSION

The epithelium, stroma, and endothelium comprise the three major anatomical layers of the cornea. Each layer has distinct and different characteristics of morphology, metabolism, and function contributing to the overall integrity of the cornea as a specialized tissue. The "separate, yet interrelated" functional theme is also found in the normal healing of corneal wounds. Basically, upon wounding, the epithelium migrates and spreads over the defect, closing the wound anteriorly, then, by mitosis and outward migration, the basal epithelial cells reestablish the normal several cell layers of protective squamous epithelium. The corneal endothelial cells spread and migrate to cover the wound, sealing the connective tissue layer posteriorly, after which endothelial control of stromal hydration and metabolism may be reinstated. The stromal layer is now faced with the responsibility of reconstruction of the normal collagen latticework and lamellar architecture necessary for overall strength of the cornea. This task is accomplished by mobilization of the sparse complement of connective tissue cells near the wound, transformation into active, proliferative fibroblasts, and with the aid of invading fibroblasts (morphologically transformed from infiltrating

monocytes), synthesizing new collagen, and mucopolysaccharide ground substances to be used in restoration of the normal corneal architecture.

Subclinical doses of beta radiation, administered to the cornea either immediately after or up to ten months prior to surgical wounding inhibit and delay this healing process. As one would expect, based on the functional differences of the different corneal layers during healing, the effects of the radiation inhibition are different for each of the three major layers of the cornea.

Epithelial function in the healing phenomenon was not completely inhibited by the beta radiation. Epithelial cells retained the ability to migrate, and to recover the injury, although repopulation of the normal several cell layers of squamified cells was delayed. The fine structure of the epithelium involved in repair of the irradiated wounds was different, however, from the epithelial fine structure found in non-irradiated corneal wounds. The most prominent differences observed in the basal epithelial cells that recover the irradiated wound were (1) a paucity of intracellular organelles, (2) a lack of rough endoplasmic reticula development, (3) enlarged intercellular spaces, and (4) a lack of basement membrane regeneration. The radiation also caused a reduction in the induced succinic dehydrogenase activity observed in normal corneal healing. Autoradiographic studies of labeled precursor uptake further showed that the radiation had reduced DNA and RNA synthesis in the epithelium. Although a decrease in DNA synthesis is not necessarily a positive indication of a decrease in mitotic activity (120), the observed

radiation inhibition of DNA synthesis in these epithelial cells is consistent with the delay in mitosis implied by the delay in regeneration of the normal several cell layers of epithelium.

The lack of basement membrane regeneration suggested that the epithelial synthetic capability was effected. This was congruous with the observed reduction in the rate of RNA synthesis, normally associated with the intracellular production of messenger and ribosomal RNA necessary for protein synthesis, and the lack of development of rough endoplasmic reticula.

The effects in the beta radiation inhibition of healing within the stromal portion of the wound were most significant in the inhibition of tensile strength development of the irradiated wounds. The beta radiation reduced the number of cells that appeared in the immediate wound area, especially the occurrence of mature, normal fibroblasts. A reduction in the DNA synthesis in keratocytes and fibroblasts was consistent with the observed lack of normal fibroblastic proliferation at the wound. Of those cells that did appear in the irradiated wounds, the ultrastructure of the fibroblast-like cells suggested that these cells were not capable of normal synthesis. The notable lack of rough endoplasmic reticula was consistent with the observations of almost total inhibition of collagen synthesis, and the reduction of sulfated mucopolysaccharide formation and the decrease in rate of RNA synthesis. It appeared that the drastic inhibition of collagen synthesis was a result of a lack of cells that were capable of synthesis, rather than a radiation inhibition of pathways.

Endothelial function in the corneal healing process was totally inhibited for some time after the cornea was wounded. Apparently, intact endothelial cells adjacent to the wound did not migrate or spread to cover the wound posteriorly. Radiation inhibition of endothelial migration lasted longer than three weeks of healing. The ten month recovery period between irradiation and wounding was sufficient to allow recovery of endothelial migratory ability.

Three-week wounds were covered posteriorly in the group that was allowed ten months recovery time. The rate of RNA synthesis in the endothelial cells was also inhibited by the beta radiation. This seemed to be correlated with the deficiency of rough endoplasmic reticula within the endothelial cells and the absence of regeneration of Descémet's membrane found in the irradiated corneas. The evidence suggests that protein synthesis was probably also inhibited in the endothelium. The radiation inhibition of the ability to expand and migrate over the wound might well have been a result of the inability of the endothelial cells to synthesize new cytoplasmic protein materials necessary for enlarging cell volume, since the mobility capacity of most cells is quite radioresistant (122).

Corneal edema is generally thought to be an index of a lack of endothelial integrity. The absence of endothelial migration in the irradiated wounds prevented closure of the wound posteriorly and prohibited reestablishment of normal endothelial control over stromal hydration and metabolism. The extensive corneal edema noted during severe inhibition of healing may well have been predominantly a result of the inhibition of endothelial function.

The inhibition of induced succinic dehydrogenase activity in the corneal wound by beta radiation could have serious implications in the production of metabolic energy within the tissue. Inhibition of succinic dehydrogenase should result in a blockage of the tricarboxcylic acid cycle, thereby compromising the metabolic energy available to the cells. In normal healing the demand for metabolic energy is greatly increased above the separate, low metabolic requirements necessary for maintaining normal corneal homeostasis (2, 29). The increased demands of the wound repair process may well put a premium on metabolic energy. A limitation of the amount of available energy could compromise the entire wound healing phenomenon. If radiation inhibition of succinic dehydrogenase activity results in a large deficiency of available metabolic energy, other metabolic sources of energy may be unable to meet the increased demands of healing. Inhibition of succinic dehydrogenase could then be the cause of other deficiencies noted in the inhibition of the healing process. Since normal corneal respiration does not operate atfull capacity (123) it is probable that the cornea has some reserve for increased metabolic energy requirements. It seems unlikely that the decrease in succinic dehydrogenase alone could account for the variety and magnitude of the changes in corneal healing observed in corneas that had been treated with beta radiation.

It is clear that many steps of the corneal healing process are affected by the beta radiation treatments. The complexity and diversity of the observed radiation effects suggest that no single

metabolic or functional step of the healing process is exclusively affected by the ionizing radiation. The variety of the radiation effects on the corneal healing system suggests that there may be some comprehensive effect of the radiation, which, in turn, affects many key steps in the normal healing process.

In normal wound healing some stimulus must initiate the multifaceted repair process. It has long been thought that some "wound-hormone" substance must be secreted or released upon injury to signal the existence of the injury and initiate repair of the damage (124). Once the repair system is activated, some management must be applied to coordinate the many, interwoven steps that lead to (1) assessment of the damage, (2) mobilization of specialized, competent cells which are necessary for debridement and synthesis of new collagen and other intercellular building materials, (3) logistic control of these newly synthesized materials, and finally (4) modulation of cell form, tissue architecture, and eventually wound contraction. This level of sophisticated management of cell function would imply the necessity for a portion of the genome to be responsible for directing wound repair processes once the genes had been alerted. This portion of the genome would probably be inactive during normal cell function and would be activated directly, or indirectly, as a result of wound-hormone action following injury.

The findings of this study would support the hypothesis that beta radiation inhibits corneal healing either by interference with the stimulus which initiates wound repair or by damaging the genetic director of the multifaceted healing process.

It has recently been suggested that initiation of the healing phenomenon might be due to some sort of derepressor acting on a repressor that normally keeps the healing process inactive yet ready for use in case of injury (125). Hormones are thought to derepress genes by complexing with a histone repressor, removing the histone, thereby activating the gene (126).

Beta radiation may effect the production, release, or action of such a corneal wound-hormone. The partial recovery noted in those corneas that had been irradiated before wounding is compatible with this possibility since it has been suggested that the concentration of active wound-hormone is directly correlated with the proliferative response observed in wound repair (125).

There is more evidence to suggest that the beta radiation elicits its effect on the genetic control system that directs the repair process. The most likely possibilities would involve either inhibition of DNA synthesis or some alteration of the DNA molecule.

If the beta radiation causes damage to the DNA molecule, thereby inhibiting corneal healing, then the partial recovery observed in these experiments would suggest that some DNA repair mechanism recognizes the damage and takes corrective action to repair the DNA molecule. Intracellular DNA repair mechanisms have been demonstrated in bacteria and are thought to exist in other cells (127); however, a DNA repair mechanism has not been demonstrated in cells of the cornea.

The observation that the radiation reduced the number of cells synthesizing DNA, more than the rate of DNA synthesis, suggests that the radiation inhibited mitosis in the cells which resulted in a subsequent lack of initiation of DNA synthesis (G_2 effect). This possibility is consistent with reduced number of cells found in the wounds of irradiated corneas and the fact that radiation inhibition of mitosis is a more radiosensitive phenomenon than inhibition of DNA synthesis (120). It is also possible that the radiation may directly inhibit the synthesis of DNA as a result of the damage sustained during the G_1 period of interphase (G_1 effect). The presence of a G_2 effect would not exclude the possibility of a G_1 effect occurring also. Either mechanism would account for the partial recovery of the wound healing capability observed when a long enough recovery period elapses between irradiation and subsequent healing.

SECTION 8

CONCLUSIONS

1. The inhibition of corneal wound healing by beta radiation, administered either prior to or immediately after surgical wounding has been demonstrated to involve many intracellular changes which have profound effects on the efficiency of the wound repair process.

2. The manifestations of the radiation inhibition were found to be separate and different for the three major layers of the cornea, which have distinctly different functions in the healing phenomenon.

The radiation inhibition of corneal healing lasted at least ten months after the irradiation treatment, even though symptoms of damage were undetectable and corneal physiology appeared normal until wounding occurred and the repair process was needed.

3. Clinical and microscopic observations showing symptoms of retarded corneal tissue regeneration, resulting from doses of 10,000 rads of beta radiation, were well correlated with a lack of tensile strength development of the wound. The quantitative lack of tensile strength in the irradiated corneal wounds appeared to be a result of an inhibition of stromal regeneration and complications of normal corneal hydration which arise from the failure of the endothelium to close the wound posteriorly.

4. The effects of the beta radiation were shown to cause an inhibi-

tion of induced succinic dehydrogenase activity that normally appears after six hours of wounding.

5. Alterations in the ultrastructure of corneal epithelium, stromal fibroblastic cells, and endothelial cells were demonstrated as part of the beta radiation inhibition of normal corneal repair.

6. A consistant lack of development of rough endoplasmic reticula and a decrease in RNA synthesis within the cells in the immediate wound area, correlated with a conspicuous lack of collagen regeneration in the wound, suggested the beta radiation has affected the protein synthesis capability of the cells. Specifically, the inhibition of collagen synthesis seemed to be a result of a lack of synthetically competent cells rather than a radiation inhibition of the specific synthetic pathway.

7. The beta radiation reduction of fibroblastic proliferation was congruous with a quantitative inhibition of DNA synthesis within the cells. The reduction in the number of cells synthesizing DNA and the amount of DNA contained in each cell nucleus suggests the beta radiation interferes with DNA synthesis by either affecting the G_1-phase of the intermitotic cycle, thereby preventing commencement of DNA synthesis by the cell (G_1-effect), or by blocking mitosis which results in a subsequent inhibition of DNA synthesis in the succeeding interphase period (G_2-effect).

8. Partial recovery of the irradiated cornea's healing capability was demonstrated when a ten month recovery period elapsed between the irradiation and surgical wounding of the cornea. This suggested the possible existence of an intracellular repair mechanism that might eventually correct the radiation damage, restoring the cornea's capability to heal itself.

9. The combined results of these studies showed that many separate, yet interrelated, steps of the wound healing process were affected by the beta radiation. The complexity and diversity of these effects suggest that no single metabolic or functional part of the repair process is exclusively affected by the ionizing radiation. It appeared that some comprehensive control mechanism was inhibited by the radiation, thus affecting many key steps in the normal healing process. The results would support the hypothesis that the radiation has interferred with some sort of wound-hormone, which is thought to initiate wound healing. There is more evidence, however, for the possibility that the radiation either inhibited DNA synthesis or damaged the DNA molecule; resulting in a faulty informational code, which could not properly direct the healing process.

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BIOGRAPHICAL SKETCH

Dennis Robert Morrison was born in Chicago, Illinois, on November 13, 1941. He graduated from Lakeland Senior High School in Lakeland, Florida, in June 1959. After his Freshman year at Florida Southern College, he transferred to the University of Florida in September, 1960.

While completing the requirements for a Bachelor of Science in Pharmacy he became interested in research. During his Junior year, he was awarded a research grant from the American Cancer Society, through the University of Florida College of Medicine, to study the "Protein Binding Effects of Certain Carcinogens" under the direction of the Graduate Faculty of the College of Pharmacy. As a result of this research effort, he was presented with the International Lunsford Richardson Undergraduate Research Award, in May of 1963.

In August of 1963, he received his Bachelor of Science in Pharmacy from the University of Florida. While enrolled part time in Graduate School, he completed his Pharmacy Internship and became a Registered Pharmacist in June, 1964.

Following a year of part time graduate studies, he resumed full time graduate studies in 1965, under a Fellowship from the Public Health Service.

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This dissertation was prepared under the direction of the chairman of the candidate's supervisory committee and has been approved by all members of that committee. It was submitted to the Dean of the College of Agriculture and to the Graduate Council, and was approved as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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